12-2013

EZH2 T416 Phosphorylation Enhances Breast Cancer Tumorigenesis

Adam M. LaBaff

Adam M. LaBaff

EZH2 T416 Phosphorylation Enhances Breast Cancer Tumorigenesis

By

Adam M. LaBaff

APPROVED:

_____

Mien-Chie Hung, Ph.D.

Supervisory Professor

_____

Dihua Yu, M.D., Ph.D.

_____

Min Gyu Lee, Ph.D.

_____

Elsa R. Flores, Ph.D.

_____

Jonathan Kurie, M.D.

_____

Dean, The University of Texas

Graduate School of Biomedical Sciences at Houston

EZH2 T416 Phosphorylation Enhances Breast Cancer Tumorigenesis

A

DISSERTATION

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

and

The University of Texas

MD Anderson Cancer Center

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTORATE OF PHILOSOPHY

by

Adam M. LaBaff, M.S.

Houston, Texas

December, 2013

DEDICATION

This dedication is to my parents, Wayne and Susan LaBaff, my grandparents, Gertrude and Ernie LaBaff and all of my family and friends that have provided me with their support and unconditional love during my time at UT MD Anderson Cancer Center, Houston, Texas.

ACKNOWLEDGEMENTS

(1) First I would to thank Dr. Mien-Chie Hung for his guidance, his support, positive attitude, and installation of team success and team science during my PhD training at MD Anderson. (2) I would also like to thank my committee members Dr. Dihua Yu, Dr. Elsa Flores, Dr. Min-Gyu Lee, Dr. Andreas Bergmann, and Dr. Jonathan Kurie Lee for taking time out of their busy schedules and for their helpful, directional, a transparent scientific support during committee meetings and one-on-one conversations. (3) To Dr. Victoria Knutson, Dr. Jon Weiner, Dr. Michelle Barton, and Lourdes “Bunny” Perez I thank you for your guidance through the on goings at GSBS. You support was professional, friendly and irreplaceable for PhD students in the GSB program. (4) I send a strong thank you to all the past and present colleagues in Dr. Hung’s research group for providing their tutelage, advice and friendship. I would like to give special thanks to Jessie Barber, Lola Small, Nancy Harkins, Stephanie Miller, Dr. Jeng Cheng, Jaw-Ching Liu, Zhenbo Han, Su Zhang, and Jian Shi for their work in keeping the administrative “wheel” turning. (5) Finally I would like to get the chance to thank my family and friends for their support. I would like to give special thanks to my friends Dr. Erika Spaeth, Dr. Aman Mann, Dr.Chia-Wei Li, and Dr Jer-Yen Yang. Through your constant communication on a daily basis you guided me, made me grow, and inspired me to work in the field of cancer research. Your influences as peers were irreplaceable.

EZH2 T416 Phosphorylation Enhances Breast Cancer Tumorigenesis

Publication No. _____

Adam M. LaBaff, M.S.

P.I.: Mien-Chie Hung, Ph.D.

Enhancer of zeste homologue 2 (EZH2) is the catalytic subunit of Polycomb repressive complex 2 (PRC2) and catalyzes the trimethylation of histone H3 on lysine 27 (H3K27Me3), to repress gene transcription. Many types of cancer stem and progenitor cells; including breast have demonstrated EZH2 to be fundamental in the biology and promoting the expansion of their cellular populations. How EZH2 regulates each of these respective CSC or tumor initiating cells (TICs) populations has been studied in a laboratory setting, but the signaling transduction mechanisms that regulate EZH2 in these CSC populations is yet to be elucidated. Phosphorylation of EZH2 by cyclin dependent kinases (CDK) has has been reported to control EZH2 epigenetic function and consequently in controlling cancer cell proliferation, invasion, and stem cell differentiation. Our group has established that EZH2 and cyclin E, the enzymatic activator of CDK2, co-expresses with clinical significance in patient biopsies of triple-negative breast cancer (TNBC) compared to normal breast cancer. Thereafter we demonstrated CDK2 phosphorylates EZH2 endogenously on residue T416 in breast cancer cell lines in a cell cycle-dependent manner. EZH2-T416 phosphorylation (pT416) enhances the ability of EZH2 to increase cell migration/invasion, mammosphere formation, and in vivo tumor growth. Tumor

growth and mammosphere formation are both mitigated with administration of CDK2 clinical trial inhibitor SNS032. Most importantly EZH2-T416 phosphorylation (pT416) correlates with poor patient survival specifically in TNBC patient biopsies paralleling the EZH2/Cyclin E IHC staining previously observed in TNBC biopsy cohorts. Therefore, we postulate pT416 to be a biomarker for aggressive forms of breast cancer, including TNBC and propose CDK2 inhibitor based therapy as a potential regimen for reducing the size of the breast cancer stem cell population and coordinately tumor size.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ATP	**Adenosine-5'-triphosphate**
BLBC	**Basal-like breast cancer**
Bmi1	**B lymphoma Mo-MLV insertion region 1**
BRCA1	**Breast cancer 1, early onset**
Cdc	**Cell division cycle**
CDKs	**Cyclin dependent kinases**
ChIP	**Chromatin immunoprecipitation**
CIP	**CDK-interacting protein 1 (p21)**
CK	**Cytokeratin**
CKI	**Cdk inhibitor**
c-KIT	**Proto-oncogene tyrosine-protein kinas Kit**
CML	**Chronic myeloid leukemia**
c-myc	**Cellular-myelocytomatosis oncogene homologue**
DNMT	**DNA methyltransferase**
E2F	**Electro-acoustic 2 Factor (Transcription factor E2F1)**
EED	**Embryonic ectoderm development**
EGFR	**Epidermal growth factor receptor**
erbB2	**Erythroblastic leukemia viral oncogene homolog 2,**
ERα	**Estrogen Receptor**

EZH1	**Enhancer of Zeste homologue 1**
EZH2	**Enhancer of Zeste homologue 2**
FBS	**Fetal bovine serum**
G1 phase	**Gap 1 phase**
G2 phase	**Gap 2 phase**
Her2	**Human epidermal growth factor receptor 2**
HOX	**Homeobox**
HPC	**Human Polycomb**
HPH	**Human Polyhomeotic**
H-Ras	**Harvey rat sarcoma viral oncogene homolog**
IHC	**Immunohistochemistry**
INK4	**Inhibitor of cyclin dependent kinase 4**
Kip2	**p57, cyclin-dependent kinase inhibitor 1C**
M phase	**Mitosis phase**
MMTV	**Mouse mammary tumor virus**
ncRNA	**Non-coding RNA**
PBS	**Phosphate buffered salin**
PcG	**Polycomb Group**
PgR	**Progesterone Receptor**
PHO	**Protein pleiohomeotic**
PHoL	**PHO-like**

PhoRC	**Protein pleiohomeotic repressive complex**
pRb	**Retinoblastoma protein**
PRC	**Polycomb repressive complex**
PRE	**Polycomb responsive element**
Rad51	**DNA repair protein RAD51 homolog 1**
RbAp46/48	**Retinoblastoma-binding protein p46/48**
Ring1A	**E3 ubiquitin-protein ligase RING1**
RNA Pol II	**Ribonucleic acid polymerase II**
S phase	**DNA synthesis phase**
SAH	**S-adenosyl-l-homocysteine**
SAM	**s-adenosyl-l-methionine**
Sirt1	**Sirtuin 1**
SMA	**Smooth-muscle actin**
SUZ12	**Suppressor of Zeste 12**
SWI/SNF	**SWItch/Sucrose NonFermentable (nucleosome remodeling complex)**
Wnt1	**Wingless-type MMTV integration site family, member 1**
YY1	**Yin and yang 1**

CHAPTER 1. INTRODUCTION

1.1 Breast cancer target therapy

The most common cancer in the United States is breast cancer and despite advances in in the clinical care an estimated 200,000 new cases with 40,000 deaths is estimated annually making it a problem of emphasis in the United States Public Health Care System[1,2]. Cytotoxic chemotherapies serve as adjuvant treatment regimens based on their inhibition of cell divisions, but the non-specific nature of these therapies produce marginal and often times adverse effects to patients[3]. As is the case with many solid tumors the current strategy of drug development has been directed to develop drugs for the inhibition of specific tumor biomolecules needed for the growth and upkeep of the tumor biology[4-7]. Targeting molecules that are unique to the function of only the tumor will kill the tumor cells and not the non-cancer cells surrounding the tumor mass via a “smart-knife”-small molecule-pharmacological approach with the hope of improving therapy specificity and efficacy.

1.1.1 Subtypes of breast cancers based on gene expression profiling

Breast cancer is a heterogeneous disease[8-11]. Currently, clinical-pathologic criteria are used to guide therapy decisions. However, anatomic pathology does not accurately define tumor biology, as tumors of the same grade and stage often behave very differently. As a result, a significant proportion of patients who received standard treatment will relapse because of ineffective therapy[12]. The use of transcriptional profiling has shown that breast cancer is a

conglomerate of at least five distinct molecular subtypes: luminal A, luminal B, Her2 positive, basal-like and normal breast-like[13]. Notably this molecular classification was done in a relatively small number of breast cancer tissues, but later validation of the five subtypes has been supported by analysis of over 1000 breast cancer tissues. From the 1000 breast cancer patient cohort straight forward and distinct stratification criteria were put forward[14-16]. For a brief example of the microarray established criteria, both luminal A and luminal B breast cancers are considered widely to be estrogen receptor positive. The Her2 positive subtype represents a group of breast cancers with overexpression or gene amplification, determined by FISH, of the Her2 receptor. The basal-like group or basal-like breast cancer (BLBC) demonstrates no ER, PgR or Her2 expression, thus is also termed triple-negative breast cancer (TNBC) in clinic, which is how nomenclature, ER-, PgR- and Her2-, was devised. The normal breast-like at times is not thought to be a breast cancer subtype and instead is thought to be contamination of normal breast tissue from the array analysis[14-16]. Overall the 4 remaining subtypes that are skeptical of being microarray artifacts are clearly associated with different clinical features listed in more detail in Table 1.

Table 1. Breast cancer subtype classification

Subtype	Markers	Features
Basal-like (80% TN)	- ER-, PgR- and Her2- -EGFR, c-kit, cyclin E, p63, alpha-SMA, P-cadherin, CK5/6, 14, 17 -high Ki67	-high grade -frequent association with BRCA1 mutation -high ridk of reoccurrence - partial chemotherapy response -no known targeted therapy
Her2 positive	- ER-, PgR- and Her2+ -GRB7 -usually high Ki67	-usually high grade -likely to involve axillary lymph nodes -high risk of reoccurrence -responsive to chemotherapy -responsive toward Herceptin and Lapatinib
Luminal A	- ER+ and/or PgR+ -Her2- -GATA3, FOXA1, LMW CK -low Ki67	-most common form of breast cancer -low grade -low risk of reoccurrence -responsive to endocrine therapy -often less responsive to chemotherapy
Luminal B	- ER+ and/or PgR+ -sometimes Her2+ -proliferation genes -higher Ki67 relative to luminal A	-variable grade - variable response to chemotherapy - usually responsive to endocrine therapy, including estrogen receptor modulation and aromatase inhibitors -responsive to Her2 modulation if Her2 present
Normal breast-like	* normal breast tissue contamination microarray artifact	

Of the classified groups, the BLBC or TNBC tumors are the most difficult tumors to treat. TNBC tumors demonstrate higher rates of therapy resistance, reoccurrence, metastasis, and therefore worse overall and relapse-free survival compared to the other subtypes.

1.1.2 Therapeutic implications of breast cancer subtypes

The molecular sub-classification of breast cancer into the 4 clinically relevant subtypes, excluding the normal breast-like as an array artifact, provided breast cancer oncologist with a biomarker based strategy for diagnosing and administering therapy[14-16]. Yes, the understanding that luminal breast cancers expressing the estrogen receptor are prime candidates for tamoxifen, aramostase, or anti-endocrine therapy or that Her2 positive breast cancers are apt candidates for Herceptin or lapatinib treatment was a clinically useful determinant in making decisions for the correct treatment for patients[17-19], but further reclassification is required. The decisions to use the above mentioned treatments and chemotherapies has been widely estimated based on tumor size, tumor grade, lymph node status, histopathological features, and the IHC staining for ER, PgR and Her2 and hasserved useful, but also has led to inadequacies and even overtreatment. The elucidation of new biomarkers for prediction of better therapy response is needed. Predictive diagnoses followed with corresponding and matching therapy treatment will reduce treatment side effects and maximize therapy efficacy with the avoidment of overtreatment[20,21]. A full gene expression profile or gene mutation status of patients and their known therapy responses would help to identify new therapeutic targets based on the

response rate according to specific treatment[22], thus going beyond the concept of overexpression breeding clinical relevance of the thus instead providing clinicians with a therapeutic marker capable of predicting patient response to drug therapy. Such a strategy would prove exceptionally useful in in ER- or basal-like breast cancer tumors in order to provide a benefit for these patients for the clinical development of treatment planning, therapeutic response, and prognosis prediction[23].

1.1.3 Basal-like breast cancer classification and available treatments

Basal-like breast cancer comprises of 15-20% of all diagnosed breast cancer[24]. At first these type of breast tumors were characterized based on their similarities with cells of the breast basal myoepithelial cell layer due to their cell similarities in composition with high molecular weight cytokeratins[25]. Further characterization required in order for these breast tumors to be determined basal-like breast cancer that additional cell markers, now termed basal-markers, such as c-KIT, alpha smooth muscle actin (SMA), EGFR, cytokeratins (CK) 5, CK6, CK 14, CK17, P-cadherin and p63[25-29]. Current classification schemes that designate breast cancer as basal-like as well as determine the classification into the three other possible breast cancer subtypes include the 70-gene assay (MammaPrint, Agendia, Netherlands), the 21-gene assay (Oncotype DX, Genomic Health, USA) and the 50-gene assay (PAM50, NanoString, USA). Of the three gene assays the PAM50 shows the most promise and since its upbringing by Parker et al. 2009 it has been trended as the most widely used array[30]. The PAM50 in combination with IHC and proliferation parameters is

proving to be an effect means to classify breast tumors into one of the four subtypes. This is important because understanding that the tumor is basal-like will avoid treatment to basal-like breast cancer patients with adulterating chemotherapies and adjuvant therapies. Although such tests allow for proper classification or at least categorization with better accuracy basal-like breast cancer remains a very difficult to treat disease with currently no target therapies available[31,32].

Triple negative breast cancer (TNBC) shares a great degree of similarity with basal-like breast cancer (BLBC) and is often referred to as one in the same because a high percentage (~80%) of BLBCs diagnose as TNBCs[33]. BRCA1 mutation carriers and premenopausal African American women are frequently triple-negative or basal-like in origin. The frequency of BLBC in African American women is twice the normal occurrence and 35 times the normal occurrence rate in germline BRCA1 mutation carriers[34]. Why African American women and BRCA1 mutation carriers are more prone to the development of BLBC is not clear. Epidemiology studies are widely emphasizing a link between African American women and BLBC therefore more investigation is needed for a conclusion and currently underway. In the case of the BRCA1 mutation carriers it has been proposed that the loss of BRCA1 or inactivation of BRCA1 wild-type gene may facilitate the outgrowth of mammary stem cell population or breast cancer stem cell population leading to the progression of tumors with stem-cell-like characteristics, a trait similar to the growth of BLBC[35,36]. Tumors of this subtype, BLBC/TNBCs, relapse at a higher frequency rate after conventional

chemotherapy and have a worse prognosis then their hormone receptor-positive luminal subtypes. The development of new systemic therapies is urgently needed as most patients with TNBC/BLBC relapse with distant metastases, and standard of care hormonal therapies and HER2-targeted agents are ineffective in this group of tumors. [28,37]Broad scopes of therapeutic agents are being actively investigated in patients with BLBC/TNBC or BRCA1-associated tumors. Increased understanding of genetic or epigenetic abnormalities involved in the pathogenesis of BLBC/TNBC, and BRCA1-associated tumors will open up new discovery for the identification of new predictive biomarkers and consequent therapeutic possibilities for these hard-to-treat breast cancers.

1.2. Polycomb Group (PcG) proteins and composition of the PRC2

The Polycomb proteins (PcG) and their functional counter parts the Triorthax proteins (TrxG), both very essential to mammalian biology, were first identified in *Drosophila melanogaster* as transcriptional repressors and activators of the Hox genes, a gene family specifying cell identity along the anteroposterior axis of segmented animals. PcG and trxG genes have also been identified in vertebrates, where they also regulate Hox genes. PcG and trxG proteins are implicated in cell proliferation, cell migration and invasion, stem cell identity and lineage control, cancer, genomic imprinting in plants and mammals and X chromosome inactivation[38,39]. It is for this appreciation of their biological functions that PcG and trxG proteins have had great research efforts pursued to elucidate their mechanisms of action.

Four different polycomb repressive complexes (PRC) have been identified in mammals, including three PRC2 variants[40,41], which can be seen summarized in Table 2. PRC1 and PRC2 can work both dependently and independently of one another to silence gene expression[42-44]. The PRC1 is thought to inhibit transcription through ubiquitination of H2A K119 via means of steric occlusion of chromatin activating components[45]. The PRC2 mechanism of transcriptional repression is invoked by methylation of H3K27 and chromatin compaction[46]. In the PRC1-dependent silencing mechanism, the H3K27Me3 mark serves to recruit the HPC chromodomain protein of the PRC1. The gene suppression is then silenced in a combination mechanism of first the PRC2 H3K27Me3 mark and then this histone modification serves as a recruiting marker for the chromodomain containing protein, Human Polycomb (HPC), a subunit of PRC1 complex, followed by further recruitment of the PRC1core complex, including the E3 ligase Ring1B for H2A K119 ubiquitination[47,48]. For the purpose of this thesis PRC1 will not be discussed in detail, but can be further reviewed in Simon, JA et al 2013.

As mentioned EZH2 is the core catalytic subunit of the PRC2. There are two related genes sharing 65% similarity that both form PRCs, EZH1 and EZH2, respectively. The tissue specificity and gene silencing mechanisms are different for each complex containing EZH1 or EZH2[49]. EZH1 is predominantly expressed in undifferentiated tissues and non-dividing or most-mitotic tissues. The gene silencing of the EZH1 complex is less dependent upon the H3K27 methylation and more mainly dependent upon chromatin compaction. EZH2 itself is

predominantly expressed in proliferative tissues and plays a significant role in maintaining the “stemness” of undifferentiated cells. The silencing function of the EZH2 complex is dependent upon the H3K27 methylation capacity of the complex, as can be seen in by the inability to silence gene expression in cells expressing the SET domain deleted variant of EZH2[49]. Despite this first report recent evidence suggests overlapping function of EZH1 and EZH2 in their gene repression pattern and silencing mechanisms therefore their interaction may be context specific.[50] Another interesting or added complexity to the understanding behind the mechanism of PRC gene silencing is that of the targeting moiety EED splice forms,in the PRC, identified in mammalian cells. EED is important for targeting the PRCs to PRC target genes and it is known to exist in 4 isoforms. For example the PRC3 contains EED isoforms 3 and 4 and can methylate H1 in vitro[51]. This could be an in vitro artifact as no biological significance has been determined for this event, but an interesting observation as EED 1 and EED2 do not possess the ability to invoke the same in vitro methylation specificity. Addition subunit association with the PRC is thought to change its targeting specificity and silencing capacity, such as histone demethylases or long non-coding RNA association[46,52]. Other post-translational modifications have also demonstrated the ability to alter PRC2 gene silencing pattern and function[53].

Table 2. Polycomb repressive complexes and their function

Complex	Components	Function
PRC1	-Ring1A,1B -PHC1-2 -BMI1 -Mel18 -CBX2-8	-H2A K119 ubiquination -antagonize RNA Pol II and SWI/SNF recruitment
PRC2	-EZH2 -SUZ12 -EED1 -JARID2 -RbAP46/48	-H3K27 methylation -H1K26 methylation in the presence of H1
PRC3	-EZH2 -SUZ12 -EED3,4 -RbAP46/48	-H3K27 methylation
PRC4	-EZH2 -SUZ12 -EED2 -RbAP46/48 -Sirt1	-H3K27 methylation - undifferentiated cell types -induced assembly with EZH2 overexpression

1.2.1 Genes targeted by PRC2 complex and its targeting mechanisms

Genome wide screening approaches have been used to examine PcG protein (i.e.EZH2) distribution in different cell systems including Drosophila, mouse, and human[44,46,54,55]. Results show that focal association points of PcG proteins highly correlate with regions methylated on H3K27, and negatively correlates with RNA polymerase II associated genomic loci[56]. The area of association was located within core promoter regions near the transcriptional start sites or in regions of known transcription factor binding elements. Many of the promoter regions targeted by EZH2 or PcG proteins were developmental genes. A consensus site was not seen in mammals for areas of EZH2 binding, but in Drosophila, specific polycomb repressive element (PRE) sequences were defined[56,57]. Additional studies in mice showed that possibly there is a significant overlap of genes targeted by the PRC2 and yin and yang protein (YY1 suggesting YY1 may help to target the PRC2 to its target loci[58], but further validation of these studies is required. ChIP-sequencing on a genome wide scale in mouse ESC showed 97% of PRC2 targets correspond to CG-rich regions or known CpG islands. This suggests either that CG-rich regions play a role in PRC2 recruitment for PRC2 targeted histone methylation at these promoters and perhaps EZH2 and its H3K27Me3 mark have the ability to later induce CpG island methylation through recruitment of DNA methyltransferases to the CpG islands[59,60].

1.2.2 The PRC2 in cancer progression: the role of EZH2 in BLBC

It is know that the aberrant activity for several PcG proteins, such as Bmi1, SUZ12, and EZH2 are implicated in the development, progression, therapy resistance, and metastasis mechanisms of several different cancers, including breast cancer[61,62]. EZH2 overexpression was originally identified to be an oncogene in lymphoma after ectopic expression to increase tumor proliferation[63]. Since this seminal finding EZH2 overexpression has been implicated in several cancer types to invoke the transformation of resident normal cells, to cause resistance to anoikis, increase cell invasion/metastasis, promote angiogenesis, induce genomic instability, cause resistance to chemotherapy, induce expansion of the breast cancer stem cell population, and increased tumorigenesis *in vivo*[64,65]. The suppressor of Zeste 12 (SUZ12), a core component of the PRC2, essential for maintaining the methyltransferase activity and stability of EZH2[66,67], has also been shown in several to increase tumorigenesis. Knockdown by shRNA of either EZH2 or SUZ12 has shown to cause reduction in tumor cells' abilities to proliferate, invade, and expand the sizes of the cancer stem cell populations[64] suggesting with the above mentioned overexpression effects a strong line of evidence that the PRC2 functions as a bona fide oncogene through enhancement of many of the cancer cell dogmas described by Hanahan and Weinberg.

Clinically EZH2 overexpression has shown to correlate significantly with tumor proliferative indexes, invasiveness, increased expression in metastatic tissue, and reduced overall survival with a higher reoccurrence rate[68-70]. Elevated

protein expression of EZH2 also correlates with poorly differentiated carcinomas[71], including breast carcinoma and was reported to be a poor prognosis marker in triple-negative and basal-like breast cancers[72-74]. In a clinical setting poorly differentiated breast tumors contain stem or progenitor-like cell populations that exhibit overexpression of basal cytokeratins, vimentin, low surface expression of E-cadherin ,and are enriched in CD44+/CD24- cancer stem cells (CSC) or breast tumor initiating cells (BTIC)[28,31,37,75], which is thought to be derived due to the EZH2 overexpression in these tumors[76]. The exact mechanism of how EZH2 provides for the advancement in tumor progression is unclear and it may not be one mechanism or silencing of one gene that causes the tumor progression. EZH2 has the capability of altering a complete mosaic of transcriptional profiling through its epigenetic transcriptional repression mechanisms therefore it may be a combination of silenced genes that leads to the EZH2-tumor promoted phenotype. Canonical examples of EZH2 silencing of tumor suppressors are currently understood, one of the most notable are the PRC2-PRC1 transcriptional repression of the tumor suppressor locus INK4b–ARF–INK4a. Both p15INK4b and p16INK4a act as inhibitors of the cyclin dependent kinases (CDKs) to control progression through the G1/S phase of the cell cycle, while ARF expression stabilizes and increases the function of P53. Thus, silencing of the INK4b–ARF–INK4a locus can promote tumorigenesis through different mechanisms[77]. Moreover the PRC2 is also known to silence the transcription of Rad51 and elevate Raf1 expression to induce genomic instability causing expansion of the breast cancer stem cell population[78], silence E-

cadherin and BRCA1 to transition cells to a more basal-like phenotype with increased metastatic potential[76,79,80], and transcriptionally inhibit Vash1 to promote tumor angiogenesis[81]. Other tumor suppressor and metastatic inhibitors that are also silenced by the PRC2 include $p57^{Kip2}$, RKIP, and tissue inhibitors of metalloproteinases[82,83].

The H3K27Me3 mark is predominantly located in the transcriptional binding sites of promoter regions and strongly correlates with the positioning of CPG DNA methylation loci. It has been proposes that by recruiting DNA methyltransferases (DNMT) to H3K27 methylated histones, via a chromodomain recognizing subunit of the DNMT complex, CPG islands are cytosine methylated DNMTs. The EZH2 H3K27Me3 mark is proposed to recruit DNMT3a and DNMT3b. DNMT3a and DNMT3b are the de dovo DNA methyltransferases needed to methylate new DNA[84-88]. Their counterpart and maintenance DNA methyltransferase, DNMT1 methylates heterchromatin. Together this provides evidence why PcG target genes are both H3K27 methylated and cytosine methylated at promoter regions in human tumors[89]. The coordination between these transcriptional repression mechanisms leads to the aberrant transient and long-term repression of PcG target genes.

EZH2 protein levels can be altered in cancer cells. The elevated expression level in turn can lead to changes in the core component composition of the PRCs. For example elevated expression of EZh2 can lead to the formation of a recently and newly characterized complex called PRC4, which core components consist of EZH2, SUZ12, EED2, and the histone deacteylates

Sirt1[41]. This altered composition of associated proteins or PRC proteins in general has the ability to affect PRC targeting to differential target gene promoter regions through altering the composition of the PRC complexation components. Altering of the normal targeting of the PRCs is hypothesized to be one way cancer cells hi-jack the PRC to promote oncogenesis[46,52,90]. Aberrant up-regulation of PRC components can be up-regulated transcriptionally by the Rb-E2F or HIF-1 alpha pathways[78,91], both common physically occurring themes in tumor biology. Additionally down regulation of microRNA-101[92,93] can also lead to the promotion of EZH2 expression. Taken together these suggest and display transcriptional, associated subunit changing, and PTM mechanisms for promoting oncogeneic-PRC signaling.

1.2.3 The PRC2: TNBC, CSC and EZH2 kinase regulated targeting

The aggressive biology of TNBC is inferred to be the result of the existence of the cancer stem cell or breast tumor-initiating cell (BTIC) population, defined by expression of the cell-surface markers CD44+/CD24-[37,94]. The BTIC self-renewal capacity creates a cellular compartment that drives tumorigenesis and generates the molecular heterogeneity of breast tumors [REF]. Shown by many independent investigators, cell populations isolated based on CD44+/CD24- cell surface marker expression demonstrate the ability to form mammospheres or tumor spheres *in vitro*. In xenograft transplantation models, as few as one hundred CD44+/CD24- cells isolated from an existing tumor can generate secondary tumors that exhibit the same phenotypic heterogeneity of the initial tumor[94]. Additionally, we and others have shown that BTIC cells are widely

resistance to most conventional therapies[94], while conventional treatment regiments, such as radiation and chemotherapies, can stimulate expansion or preferentially select for BTICs [94]. Thus, translation of recent basic biology research on BTIC led to active clinical investigation of a broad scope of targeted therapeutic agents in patients with TNBC tumors for targeting the BTIC population [94,95].

Stem cells are functional units of growth, repair and regeneration after tissue damage or loss. The stem niche protects stem cells from depletion over the course of the lifespan by providing a specialized microenvironment. They are generated and maintained in what is thought to be either symmetric or asymmetric division depending on the harboring tissue. As the lifespan of the organism increases the stem cell niche supplies the stem cells to the specific tissue areas needed to maintain tissue specific homeostatic balance. It is here that tumor or tumor stem cells may originate from cells that undergo “malignant reprogramming” driven by genetic and epigenetic events [96-98]. The cancer stem cell hypothesis augments this argument by stating that malignant deregulation occurs in the breast stem or progenitor cell compartment, thus altering the self-renewal program and switching the normal upper level lineage of mammary gland cells to BTICs of a progenitor or stem cell origin [94]. This hypothesis may be supported in a secondary model explaining the generation of cancer stem cells through a dedifferentiation process of differentiated cells into cells that are similar to progenitor or stem cells that then can reside in the stem niche as cancer stem cells once they have been reprogrammed to the upper lineage cell type. Studies

show that BTIC gene expression profile is similar to that of the mammary gland during embryogenesis and early development thus BTIC are genetically similar in their gene expression profile to upper lineage breast cells. Epigenetic regulatory mechanisms are connected in a network that permits a synergistic mosaic regulation of specific genes, such as transcriptional programming of embryonic and adult stem cells that controls their self-renewal and differentiation. These systems indicate that generation and maintenance of BTICs can occur via independent or parallel mechanisms. Epigenetic mechanisms may switch these BTIC markers and genes “on” and “off” to generate phenotypically distinct cell populations with survival advantages that contribute to tumor initiation and progression[99-101]. Improved understanding of genetic or epigenetic abnormalities involved in the pathogenesis of BTICs will open up new therapeutic possibilities for these hard-to-eliminate cells, and bring new hope to breast cancer patients. Below, we describe some of the crucial players that regulate epigenetic machinery and propose that by better understanding their function specifically in BTIC compartment of TNBC, we will identify new therapeutic targets for treatment of this deadly cancer.

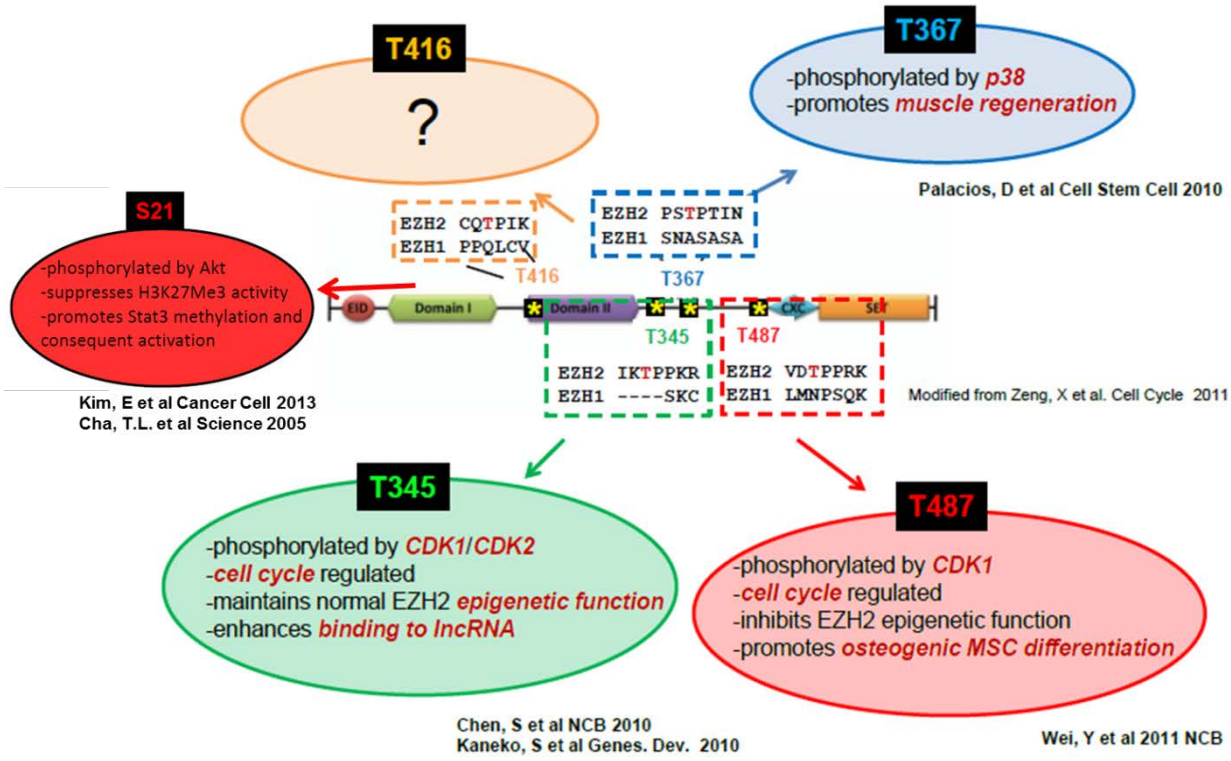
The Polycomb and Trithorax groups are transcriptional repressors and activators that function in multimeric complexes that interact with chromatin or histones, leading to repressed or activated state of gene expression, respectively [REF]. The Polycomb Repressive Complex 2 (PRC2) initiates repression of gene transcription in breast and many other types of cancer via tri-methylation of histone 3 (H3) lysine 27 (H3K27Me)[102]. The core components of the PRC2

complex necessary for its H3K27 tri-methylation function are Embryonic Ectoderm Development (EED), Enhancer of Zeste 2 (EZH2), Suppressor of Zeste 12 (SUZ12), and Retinoblastoma-associated proteins 46 and 48 (RbAp46/48) histone-binding proteins (REF). EZH2 serves as the catalytic methyltransferase component of the PRC2, adding methyl groups to H3K27 of its target gene promoters, silencing them[102]. Although EZH2 expression and H3K27 tri-methylation are associated with an array of cancer types, little is known about the molecular mechanisms that control EZH2 itself, such as what extra- or intracellular signals induce changes in PRC2 composition or activity, how EZH2 is targeted to specific promoter regions, or how these alterations promote aggressive tumor phenotypes in target tissues. Because of its gene silencing properties, EZH2 is one of the key components involved in maintaining self-renewal, pluripotency, and differentiation of embryonic and adult stem cells[96-98,100]. Similarly, EZH2 is essential for tumor stem cell biology. We showed that expression of EZH2 sustains a reversible and undifferentiated stem cell-like phenotype in breast cancer cells and can contribute to their expansion through up-regulation of RAF1-β-catenin signaling axis [78]. Furthermore, pharmacological inhibition of EZH2 inhibits cancer stem cell self-renewal, reduces expression of stem cell surface markers, and inhibits in vivo tumor initiating capacity of various tumors. Elevated protein expression of EZH2 correlates with poorly differentiated breast carcinomas and was reported to be a poor prognosis marker in triple-negative and basal-like breast cancers[74]. Clinically, poorly differentiated breast tumors contain stem or progenitor-like cell populations that exhibit

overexpression of basal cytokeratins, vimentin, and low surface expression of E-cadherin, which were all previously shown to be enriched in CD44+/CD24- BTIC cells[36,71,96-98,100]. Substantial studies highlight the role of PcG proteins, with an emphasis on EZH2, to maintain stemness by repressing lineage differentiation genes[36,96-98,100]. Taken with the above this suggests EZH2 and PcG proteins might contribute to tumorigenesis through the support and maintenance of cancer stem cells. The concept provides an interesting hypothesis for why tumor cells can appear to proliferative for a potential amount of indefinite time with perplexing tumor cell heterogeneity. This working model is very similar to the self-renewal of stem cells in tissue homeostasis or more specifically in this case, in tumor biology via cancer stem cells homestasis. As described above, EZH2 expression and H3K27 tri-methylation are well studied in cancer, but knowledge of kinases that regulate EZH2 is limited. A few precedent EZH2 threonine (T) and serine (S) phosphorylation examples have been elucidated, demonstrating that EZH2, and in hand the PRC2, can be directly regulated through kinase phosphorylation, resulting in increase or decrease of EZH2 activity. Specifically, cyclin-dependent kinases 1 and 2 (CDK1 and CDK2) both have been demonstrated to phosphorylate EZH2 at T345 and promote gene silencing, cell proliferation, and cell invasive properties elicited by EZH2[103,104]. P38 kinase has also been shown to activate gene silencing function of EZH2 via T367 phosphorylation and subsequent promotion of muscle stem cell proliferation[105]. Conversely, CDK1 and Akt can both inhibit gene silencing functions of EZH2 via its phosphorylation at T487[106]and S21[107], respectively, thereby inhibiting H3K27

tri-methylation of PRC2 target genes, and reversing EZH2 oncogenic functions. Therefore, we hypothesize that inhibition of “EZH2 activator” kinases or activation of “EZH2 inhibitor” kinases is a direct way to reduce EZH2 function in BTICs. Known phosphorylation sites and their biological functions are summated in Figure 1.

Figure 1. The biological significance of EZH2 phosphorylation

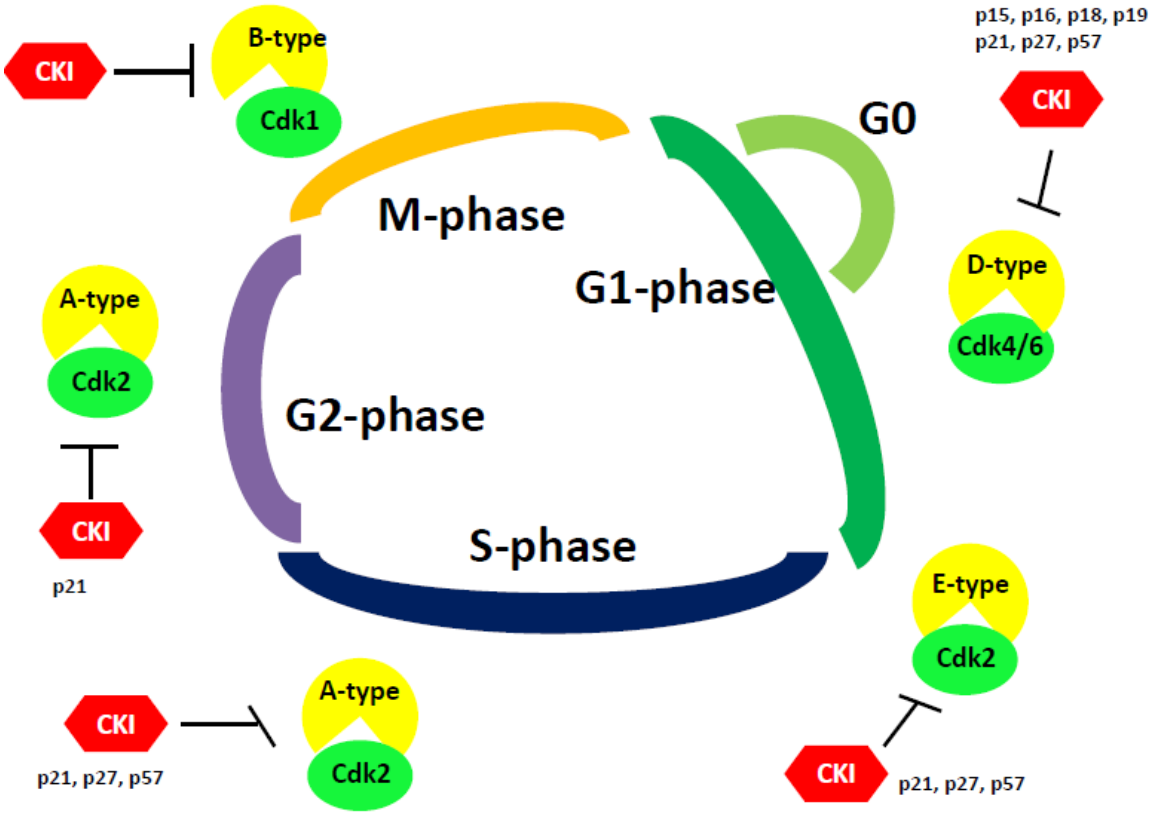


1.3 Cyclin dependent kinase

The cell cycle regulation model was derived from studies in yeast using cell division proteins. It was the first comparative model as there exists only one cell cycle dependent kinase in yeast, Cdc2 in *Schizosaccharomyces pombe* and Cdc28 in *Saccharomyces cerevisiae.*In either *Schizosaccharomyces pombe* or *Saccharomyces cerevisiae.* The cell cycle regulated by the cyclin dependent kinases promotes cell cycle progression by pairing with distinct cyclins at different stages of the cell cycle. Mammals have many cell cycle dependent kinases (CDK) that are grouped in family my gene homology, some are responsible for regulating cell cycle events through cyclin pairing and others are transcriptional CDKs. The mammalian cell cycle consists of four phases: (1) for DNA synthesis called S-phase (2) the mitotic phase called M-phase, (3) the first gap phase called G1-phase (4) the second gap phase called G2-phase. When resting cells also known as quiescent cells that rest in (G0) receive a mitogenic signal cell cycle progression occurs via distinct CDKs being sequentially activated by their partnering cyclins to drive the cell through interphase (G1, S, and G2) and the mitotic phase (M). The first cell cycle activation event is the induction of cyclin D expression (D1, D2, and D3) which commitently partners with and activates CDK4 and CDK6. Further progression occurs through CDK4 and CDK6 phosphorylation of pocket proteins RB, p107, p130 and expression of cyclin E (E1 and E2), previously transcriptionally repressed by the Rb/E2F transcriptional. The increase of cyclin E in late G1 further phosphorylates and inactivates the pocket proteins via cyclin E coupling with and activating CDK2.

This allows passing through the first checkpoint in late G1 before entering S-phase. After passing this checkpoint the cell cycle is irreversible. RB is held in its hyperphosphorylated status through cyclin A coupling and activation of CDK1/2 in addition to cyclin B coupling with and activation of CDK1, with all activations maintained until the end of M-phase. The passing on of CDK2 activation by coupling with A-type cyclins, specifically cyclin A2, and activation of CDK1 promotes the cell cycle and biological events from late S to G2 before causing the initiation of mitosis. The cyclin A proteins are degraded with the breakdown of the nuclear envelope. Then cyclin B coupling and activation of CDK1 at the G2/M cell cycle mark drives cells through mitosis and back to the beginning of the cell cycle for activation of another cell cycle process[108,109]. A schematic of the on goings of the cell cycle can be seen in more detail in Figure 2.

Figure 2. Cyclin and CDK activation throughout the course of the cell cycle



1.3.1 Cyclin dependent kinases (cdks) and cancer

Deregulation of the cell cycle is a predominant and frequent feature in human cancer. Cancer cells commonly undergo (1) unscheduled proliferation, (2) genomic instability through increased DNA mutations and chromosomal aberrations, (3) chromosomal instability seen in changes in chromosome number. CDKs are frequent targets for aberrant cancer cell signaling[108]. The up-regulation of their activity originates from genetic and epigenetic deregulation of both CDKs and their activator or inhibitor proteins and upstream mitogenic signaling. The aberrant activation or loss of pRb is one down stream example resulting through CDK hyperactivation, such pRb loss has been demonstrated as an oncogenic event in many cancers[108,110]. CDK hyperactivation of can be initiated through abnormal expression of D (CDK4/CDK6) or E-type cyclins (CDK2) or loss of p16INK4a (including mutation based acquired insensitivity to p16INK4a) possibly, as elduded to before, through EZH2-dependent gene silencing. Abnormal overexpression of cyclin E and downregulation or loss of CDK2 inhibitors p21 and p27 are also frequently observed in many tumors, but interestingly follow-up genetic experiments indicate that CDK2 does not play a significant role in cells lacking p21 or p27.Iit is thought that because p21 and p27 can also inhibit CDK1 that in these tumors tumorigenecity is due to deregulation of CDK1 and not CDK2 thus demonstrating a role for CDK1 activation driving tumorigenecitiy. Cyclin E overexpression does still drive the tumor development and progression of highly aggressive tumor phenotypes. In the case of the cyclin E overexpression tumors, both long and short forms, the tumors are dependent

on CDK2 function. This demonstrates that aberrant activation of each respective CDK has the ability to promote tumor development with proposed different driving mechanisms and tumor promotion under different contexts For example emerging studies suggest cancer cells may have specific utilization for different CDKs. CDK4 is dispensable for mammary gland development, but is needed for promoting breast cancer development dependent upon Erbb2, Hras or Myc oncogenes[108,110].

The constitutive and aberrant activation of CDKs has the ability not only to contribute to increased tumor cell proliferation but also to genomic instability[108,110]. In turn alteration of DNA damaging signaling pathways and response, and mitotic check points, frequently leads to elevated CDK activity. Despite the complexities and difficulties they cause for developing treatment strategies and drugs based on CDK context specificities and structural similarities. CDK based therapy strategies have treatment potential. Design of the treatment regimen plans should take into consideration the CDK biology of the tumor being treated.

1.3.2 CDKs, adult normal stem cells and cancer stem cells

Adult stem cells are in a constant fluctuation of steady-state dormancy and activation phases based on the tissue niche's homeostatic need to repair damaged tissues or to provide growing tissue with differentiated lineage specific cell supply. The fluctuation between these states as well as the production of new stem cells through symmetric or asymmetric division requires delicate control. Releasing stem cells from their quiescent state to enter the cell cycle

without checkpoint controls would exhaust the stem cell population and inhibit the stem cell exhausted tissue from repairing cell damage or from providing new growth capabilities. Thus, cell cycle regulation is essential for maintain stem cell homeostasis in native tissues[96-98]. The balance between CDKs and their inhibitors have been determined to be crucial for maintaining both stem cell and progenitor cell populations. CDK deficient activity may cause for a limited stem cell population incapable of tissue upkeep while aberrant activity may induce expanded stem cell populations leading to genomic instability, genetic or epigenetic hits and consequent tumorigenic events. Studies demonstrating CDKs' roles in stem cell biology are mentioned below. For example animal studies demonstrate redundant effects of CDKs in maintaining the self-renewal capacity of neural progenitor cells. In this model, CDK2 deficiency was shown to cause differentiation an effect synergized through pharmacological inhibition of CDK4. CDK influence on stem cell biology can be seen in other adult stem cell models exemplified through regulation of known CDK inhibitors or CDK inhibitor (CKI) protein familes, INK4 and Cip/Kip. Embryonic stem cells also have their renewal-capacty tightly regulated by CDK activity, CDK2 specifically. Taken together this suggests multiple CDK involvement occurs in multiple stem cell models, including embryonic, adult, and progenitor cells. These parallel systems provide model for tumor stem cell and progenitor cell biology therefore put forward a suggestive role of CDK involvement and significance in upper lineage tumor cell homeostasis[96-98,108,110]. Furthermore, the CKI protein families are tightly regulated by stem cell maintenance and are involved in promoting

signaling pathways of Notch and TGFβ/SMAD, known stem cell promoting pathways, suggesting a true hiearchy of cell control of CDK activity in the stem cell niche. The deregulation of this checks and balance system may cause deviation of normal stems to later become cancer stem cells through aberrant signal transduction mechanism. Once the cancer stem cells reside in the tissue stem cell niche they benefit from the niche's provided protection and can replicate indefinitely to arm one cancer cell with the molecular complexity, heterogeneity, and support to promote tumor development. CDKs may in fact present a therapeutic intervention point to target the cancer stem cell population in certain cancer types with dependency on that specific CDK.

CHAPTER 2. STATEMENT OF OBJECTIVE

BRCA1 inherited mutation carriers have been shown to be almost %90 basal-like breast cancer (BLBC) in tumor origin. This is unique because very little is known, other than BRCA1,about what mechanism drives the development of BLBC. Often BRCA1(albeit possibly set apart from other basal-like breast cancers that do not harbor the BRCA1 mutation) is used as the best known model system for basal-like breast cancer. Suggestive of similarities between BRCA1-derived BLBC and sporadicBLBC, both are predominantly triple-negative, as can be seen by their lack of expression for the estrogen, progesterone, and HER2 receptors in IHC immunohistochemical staining and similar in their gene expression profiles. As mentioned previously nearly %80 of BLBCis diagnosed as TNBC.

The function of EZH2 recently has been linked to BLBC or TNBC through reports showing EZH2 correlations with undifferentiated breast cancer and as poor prognostic marker in BLBC. Moreover EZH2 was found to decrease BRCA1 expression, observed to be overexpressed in human tumors and mouse tumor models that are BRCA1-deficient, and dependent for the survival of BRCA1-deficient cancer cells. Knowing BRCA1 loss drives cancer by promoting the expansion of aberrantly tumorigenic upper lineage breast cancer cells with similar genetic profiles as sporadic BLBC the above mentioned evidence is suggestive of EZH2 playing a role in modulating the biology of BLBC by one of two ways (1) by maintaining cells in a non-differentiated state or (2) through a process of de-differentiation most likely through EZH2-PRC2 dependent function.

Each model strikes similarity to the proposed model for the generation of stem cells or breast cancer stem cells, which in 2011 Chang et al. demonstrated EZH2 directs the expansion of the breast tumor initiating cell (BTIC) population.

Cyclin E/CDK2 also relatedly demonstrates involvement in BLBC. High cyclin E expression is observed in BRCA1 mutation carriers and the high expression Cyclin E negatively correlates with poor survival of the BRCA1 mutation carrier patients. CDK2 being the only known catalytic partner of the Cyclin E/CDK2 complex demonstrates that the elevated cyclin E levels is also associated with increasing CDK2 activity, which is further supported by not only high expression level of cyclin E in BRCA1-derived BLBC, but also the loss of the CDK2 inhibitor protein, p27, in these tumors. The elevation in CDK2 activity being important for basal-like breast cancer can most directly be observed in an MMTV mouse model expressing a constitutively active CDK2 and its ability to generate mammary tumors with a basal-like signature or component. This data suggest that CDK2 activity may play a biologically significant role in BLBC.

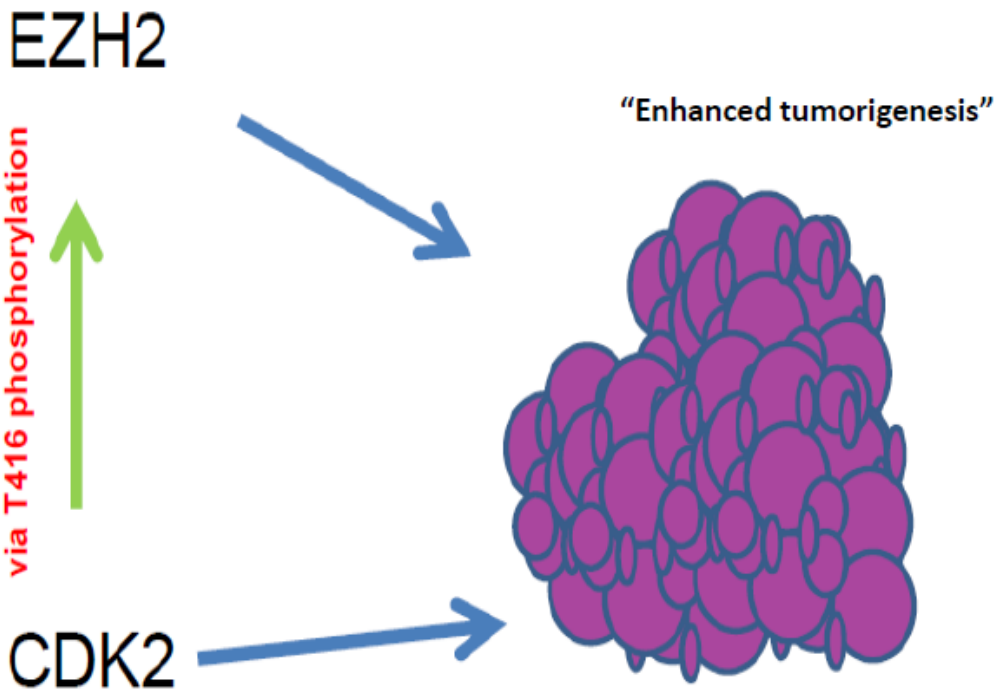
EZH2 has been shown to be transcriptionally Rb/E2F up-regulated duringthe cell cycle. EZH2 peak expression during the cell cycle occurs at the G1/S transition checkpoint. At this same stage of the cell cycle CDK2 activity also peaks due to a rise in cyclin E expresson and has continuous activity until the G2/M transition via complexation with A-type cyclins. EZH2 has previously been shown to be regulated by CDK phosphorylationin a cell cycle dependent manner on residues T345 and T487. This suggests that PRC2 activity, including EZH2, can be modulated through deregulation of the cell cycle, specifically through

CDK2 activity. Such aberrant PRC2 activity is synonymous with maintaining undifferentiated cell states similar to BLBC gene signatures, increased pools of cancer stem or progenitor cells and coincidently aggressive tumor traits representative of BLBC. Understanding the underlying signaling events that generate the development of or maintain the biology of BLBC will provide insight into the development of rationalized targeted therapies to for mammary oncologist to provide for BLBC patients

Rationale

Basal-like breast cancer is the most difficult to treat breast cancer sub-type with very few adequate biomarkers available to predict patient survival or therapeutic response. . It is known that independently of each other both cyclin E and EZH2 overexpressi in basal-like breast cancer and are markers of poor prognosis. Further linking the two proteins, EZH2 expression level has also been reported to be highest during the G1/S phase of the cell, which coincidently is the cell cycle stage where cyclin E/cdk2 activity is the most robust. Knowing previously that EZH2 has been implicated in the downregulation of BRCA1 and E-cadherin, known events leading to the development of a more basal-like breast cancer phenotype, and that EZH2 can be regulated by CDK phosphorylation, we asked if cyclin E/cdk2 may regulate EZH2 to (1) modulate EZH2 function and (2) increase breast cancer tumorigenecity in a manner similar to a tumor representative of basal-like breast cancer and (3) does the EZH2 phosphorylation correlate with patient survival trend.

Figure 3. Triangle hypothesis of cyclin E/Cdk2 regulation of EZH2 function



Hypothesis

The cyclin E/Cdk2 complex phosphorylates EZH2 at evolutionary conserved CDK2 phosphorylation consensus site causing an increase in the tumorigenecity of breast cancer cells capable to generate a more aggressive tumor phenotype with enhanced basal-like breast cancer characteristics. We propose this occurs through modulation of EZH2 canonical oncogenic functions.

Below are the specific aims for testing the above mentioned hypothesis:

Aim 1: Investigate the pathological correlation between cyclin E/CDK2 and EZH2 in triple negative breast.

Cyclin E/CDK2 and EZH2 each have recently been shown to correlate with poor patient prognosis in basal-like breast cancer, but the co-expression, suggestive of modulation between the two, has yet to be determined. To investigate if there is clinical significance in triple-negative breast cancer (TNBC) between E/CDK2 and EZH2 cyclin tissue microarray cohorts of TNBC and non-TNBC biopsies will be evaluated for cyclin E and EZH2 expression by immunohistochemical staining. Potential cyclin E/CDK2 protein-protein association will be evaluated endogenous using co-immunoprecipitation in candidate TNBC cell lines and *in vitro*. Phosphorylation of EZH2 serine or threonine amino acid residue will be mapped to EZH2 functional domains based on using *in vitro* kinase mapping assay. Identification of the serine or threonine residue *in vitro* will be confirmed by generation of a custom phospho-antibody.

Aim 2: Investigate the clinical significance of EZH2 phosphorylation.

In order to investigate the clinical significance of the EZH2 serine or threonine phosphorylation sites, a custom antibody will be generated recognizing the phosphorylation site suitable for IHC. Tissue microarray cohorts of TNBC and non-TNBC biopsies will be immunohistochemical stained using the custom phospho-EZH2 antibody and survival data will be compared with EZH2 phosphorylation levels to determine if patient overall survival decreases with increasing EZH2 phosphorylation levels. Supportingly, cyclin E expression levels will be analyzed to determine if Cyclin E elevated expression correlates with elevated EZH2 phosphorylation levels.

Aim 3: Elucidate the cell cycle dependency and biological function of cyclin E/CDK2 phosphorylation of EZH2.

To examine if EZH2 phosphorylation is cell cycle dependent, TNBC candidate cell lines will be synchronized in G1/S of the cell cycle or G2/M of the cell cycle using double-thymidine or nocodazole block, respectively. After release from cell cycle synchronization, phosphorylation of EZH2 will be monitored using SDS-PAGE analysis over a 14 hour window (i.e via immunoblot with the custom phosphor-EZH2 antibody). Examination of cells held in G1/S or G2/M compared with unsynchronized cultured TNBC cells will determine if EZH2 phosphorylation is enhanced in either specific stage of the cell cycle. EZH2 has been implicated previously to TNBC in clinical patient samples and in the expansion of the BTIC population [REF], but how this alters the functional phenotype of TNBC or affects BTIC in TNBC has yet to be determined. To

evaluate this role of EZH2 phosphorylation stable TNBC cells lines using lentivirus infection will be generated in each of the respective TNBC cells consisting of vector, EZH2-wild type, and the threonine phosphorylation site mutated to alanine. Functional *in vitro* assays for canonical EZH2 oncogenic function such as (1) cell proliferation using Brdu (2) migration/invasion using boden chambers and matrigel coated boden chambers (3) anchorage independent growth using soft-agar, and (4) angiogenesis hMEC tube formation (5) BTIC regulation using mammosphere assay will be used to compare the panel of the abovementioned cell lines to elucidate whether the role of phosphorylation plays an enhancing or diminishing role in regulating the canonical oncogenic functions of EZH2. In addition mammospheres generated in the above mentioned panel may not demonstrate change in $CD44^{hi}/CD24^{lo}$ cell populations so alternate cell surface markers for tumor progenitor cell markers will be assed including EPCAM, CD49f, and CD133. In addition aldefluor assay will be used to determine the relative level of ALDH1 activity thus the relative level of tumorigenic capacity of each of the respective TNBC lines in the *in vitro* spheres to further characterize the cell marker studies. Similarly, in order to delineate direct role of EZH2 phosphorylation from CDK kinase function or the phosphorylation causing direct loss of global EZH2 function the above mentioned functional assays 1-5 will be performed in TNBC cells that have had established lentiviral knockdown of the CDK of interest determined in Aim 1 and, independently, EZH2 lentiviral knockdown. The *in vivo* tumorigenic capacity of EZH2 phosphorylation, whether it be diminishing or enhancing of wild-type EZH2,

will be determined by injection of $1.0x10^6$ TNBC cells from the panel described above, including the shLuc, shCDK and shEZH2 controls, into the mammary fat pad of female nude mice. Each respective cohort will consist of 10 mice with each mouse of the cohort injected with the same number of the indicated cell line; (1) shLuc (2) shCDK (3) shEZH2 (4) vector (5) EZH2 wild-type (6) EZH2-T/A. The *in vivo* growth of the tumors will be determined by tumor palpitation to determine tumor volume. In addition tumor weight and size will be determined.

Aim 4: Determine potential for designing a therapy regimen for reducing the effects gained through cyclin E/CDK2 phosphorylation of EZH2

As TNBC is a very difficult to treat disease with currently very limited target therapies available, gathering an understanding of molecular mechanisms driving the aggressive nature of TNBC tumor development provides a venue to develop targeted therapies against such said pathways for better treatments of TNBC. To determine if clinical trial drugs such as CDK2 inhibitor, SNS032, or EZH2 specific inhibitors, such as GSK1206, have the ability to inhibit the development of TNBC tumors a tumor sphere assay will be developed by culturing TNBC cell lines in sphere promotion forming media. Because spheres represent the functional perspective of tumor initiating cells such a sphere assay will model cells with the potential mimic of *in vivo* therapeutic resistance. If the therapy regimens works in a tumor sphere assay it should be applicable later in more rigorous pre-clinical therapy models. Efficiency of the therapy administered, whether SNS032 or GSK1206 will be determined by counting the relative number of spheres before treatment compared to the relative number of spheres after

treatment and referenced with a sphere viability index determined by staining spheres with proliferation markers such as BRDU or MTT cell stain applied in culture.

CHAPTER 3 MATERIALS AND METHODS

3.1. Antibodies, reagents, chemicals, and drugs

Antibodies from their respective places of purchase are mentioned below. EZH2, H3K27Me3, Histone 3, Cdk2, phosho-CDK2, NPM, or phospho-NPM was purchased from Cell Signaling. Cyclin E and Cyclin B1 antibodies were obtained from Santa Cruz and Neomarkers respectively. FACS antibodies were from BD Biosciences excluding the EPCAM FACs antibody, which was purchased from Biolegend. Protein A, protein G, and glutathione Sepharose beads used in immunoprecipitation were ordered from Amersham Biosciences. Cell culture supplements such as Heparin and Cortisozone were used in mammosphere culture assay were from Stem Cell Technologies Inc. Polybrene for lentiviral transduction into cultured cell lines was purchased from Millipore. Assays for measuring cell migration/invasion coated with basement membrane in the case of invasion plates were purchased from BD biosciences. Soft agar assay plates were made in-house using Bio-Rad agarose with low melting point. All other tissue culture plates were purchased from Corning. [γ^{32p}] ATP (4500 Ci/mmol) was from MP Biomedicals. Small molecule inhibitors for CDK2 or EZH2 such as roscovitine or DzNep A were purchased from Cayman Chemicals. CDK2 inhibitor, SNS032, was purchased from Selleck Chemicals and EZH2 inhibitor GSK343 or GSK126 were purchased from GlaskcoSmithKline.

3.2. Cell culture

Cell lines used were all purchased from ATCC and were subjected to and validated using DNA fingerprinting. Cell lines used included: MCF-7, a human

mammary adenocarcinoma cell line isolated from pleural effusion; T47D, a human mammary ductal carcinoma cell line isolated from pleural effusion; ZR751, a human mammary ductal carcinoma cell line isolated from ascites; BT-549, a human mammary ductal carcinoma originated cell line; MDA-MB-231, a human mammary adenocarcinoma cell line from isolated from pleural effusion HS578T, a human mammary carcinoma originated cell line; and 293T, a human embryonic kidney cell line. Cell lines were cultivated in Dulbecco's Modified Eagle's Medium/F12 (1.5 g/L Glucose) or Dulbecco's Modified Eagle's Medium supplemented (4.5 g/L Glucose) with 10% heat-inactivated fetal bovine serum (FBS), Penicillin/Streptomycin (100U, 100μg/ml) at 37^{o}C in a humidified atmosphere with 5% CO_2. Neomycin-resistant stable cell lines were kept in G418 (500μg/ml) in addition to above specified conditions. Puromycin-resistant stable cell lines were kept in puromycin (2.5μg/ml) in addition to the above base-line culturing conditions.

3.3. Transfection

1 x10^{6} cells were seeded on each plate for transfection in 100mm culture dish 24 hours before transfection. A 1:1 (w/v) ratio of plasmid DNA and to liposome were mixed and diluted in 200μl OPTI-medium at room temperature for 30mins after gentle vortexing. The full serum medium in each dish was changed to 3m OPTI Medium 30 mins before adding transfection mixture. The plasmid DNA:liposome mixture was then added into the cell culture dish with gentle side-to-side agitation for mixing. After 4-6 hours of incubation in the tissue culture incubator,

the medium was changed back to normal 10% FBS medium conditions and 48 hours later the transfected cells were used for experiment or harvested.

3.4. Clonal stable line selection

pCDNA3 constructs of 3xmyc-EZH2 wild type (3XMycEZH2-WT), 3xmyc-EZH2-T416A (3XMycEZH2-T416A),T416A and), 3xmyc-EZH2-T416D (3XMycEZH2-T416D) were transfected into MCF-7 or MDA-MB-231 cells as aforementioned. After 48 hours 1 $x10^5$ cells were subcultured into complete media containing 500μg/ml G418 in order to provide for clonal selection. Approximately 2 weeks later distinct cell colonies were isolated, and expanded as representative single-cell pools. These single-cell pools were tested for EZH2 expression and cryopreserved at -80C.

3.5. Western Blot

3.5.1. Cell lysis

Cell culture dishes were washed with cold PBS buffer 2X and then lysed with cold modified RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% Triton-X100, 0.1% SDS, 50 mM Tris at pH 7.5, 25 mM NaF, 2 mM Na3VO4, 5 mM PMSF, 2 μg/ml aprotinin). Cells were then harvested with cell scraper and this early process whole cell lysate was sonicated briefly for 30sec 2X at low power. Lysates after sonication were then “cleared” via centrifugation. Lysate concentration was determined by BCA protein assay (Pierce).

3.5.2. Immunoblotting

Equal amounts of total protein in equal volumes were diluted with 6x SDS-DTT, denatured by boiling for 10min at 100^oC, loaded into 6-12% SDS-polyacrylamide

gels (SDS-PAGE). Gel electrophoresis was ran at slow speed of 80V or fast speed of 180V. Electrotransfer was then performed onto PVDF membranes on ice at 0.300MA for 90min. The PVDF membranes were then blocked with either 5% bovine serum albumin (BSA) or non-fat milk dissolved in PBS for 1hr at room temperature. Blocking buffer was then washed to avoid particulate build up on the PVDF membrane by washing in PBS for 15min at room temperature. Specific primary antibodies were added at dilutions specified by the commercial antibody provider overnight at 4C. After overnight incubation, PVDF membranes were washed for 30min in PBS at room temperature then incubated with species specific horseradish peroxidase-conjugated (HRP) secondary antibody at a 1:5000 dilution for 1hr at room temperature. Following secondary antibody incubation PVDF membranes were washed in PBS for 1-3hr at room temperature or until specific band resolution was as desired in reference to background signal. The immunoblots were incubated with enhanced chemiluminescence (ECL) kit solutions after ECL1 and ECL2 mixing for 2min. Visualized signal was detected by developing on autoradiography film.

3.6. Immunoprecipitation

Immunoprecipitation reactions were carried out using an antibody to protein lysate ratio of 5ug:1mg (i.e. 5ug EZH2 antibody). Whole cell lysate was first “cleared” for non-specific interaction with sepharose beads via a 4-6hr incubation at 4C. Whole cell lysates were then subjected to incubation with target antibody overnight at 4C following next day incubation with 50ul of protein A or protein G per 1mg whole cell lysate for 4-6hr at 4C. The immunoprecipitated complex was

washed five times with PBS, suspended in equal volume of 2X SDS-DTT protein sample buffer, and denatured at 100C for 10min. SDS-PAGE and immunoblot were then used to analyze interacting proteins

3.7. Kinase assay

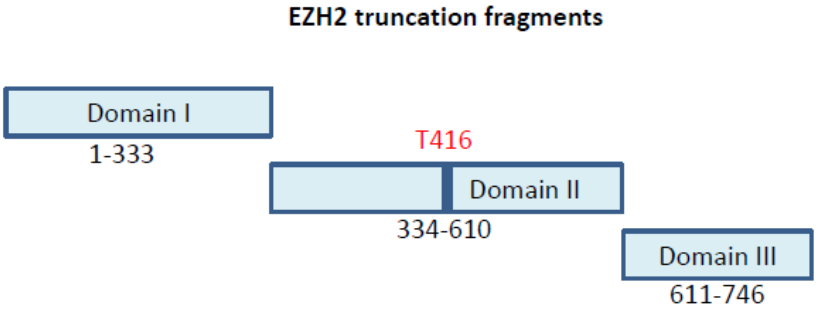
3.7.1. EZH2 truncation cloning

Three EZH2 truncations (domain I, domain II, domain III) were made in order to express EZH2 truncation fragments in GST N-terminal fusion protein format. EZH2 GST-N-terminal protein fragment domain I was from amino acid residues 1-333, domain II from amino acid residues 334-610, and domain III from amino acid residues 611-746. cDNA coding regions for the three respective domains were subcloned in pGEX-6P1 between restriction digest sites of BAMH1 and Xho1. Primers used for subcloning can be seen in Table 4.

3.7.2. Site-directed point mutatgenesis of GST-EZH2 truncation fragments

Site-directed point mutatgenesis was performed on the EZH2 cDNA mentioned in 3.6.1. Domain II subcloning primers were then used to PCR amplify domain II cDNA for later t4 ligase mediated ligation into the PGEX-6P1.

Table 3. Primers for constructing GST-EZH2 deletion fragments and T416 mutants.



GST EZH2 (1-333) 5'-CGGGATCCTGGGCCAGACTGGGAAGAAATCTGAGAAG-3' 5'CCGCTCGAGTCACTCCTTTGCTCCCTCCAAATG-3' **GST EZH2 (334-610)** 5'- CGGGATCCTGTTTGCTGCTGCTCTCACCGC-3' 5'- CCGCTCGAGTCAGGAGCCCCGCTGAATACTGC-3' **GST EZH2 (611-746)** 5'- CGGGATCCTCAAAAAGCATCTATTGCTGGC-3' 5'- CCGCTCGAGTCAAGGGATTTCCATTTC-3'
T416A mutagenesis primer 5'- GAAGCAAATTCTCGGTGTCAAGCACCAATAAAGATGAAGCC-3' **T416D mutagenesis primer** 5'- GAAGCAAATTCTCGGTGTCAAGATCCAATAAAGATGAAGCC-3'

3.7.3. IPTG induced *E.Coli* expression of GST-EZH2 protein fragments

GST-EZH2 truncation fragments were expressed and extracted from BL-21 competent E. Coli cells. Transformed BL-21 cells were grown on ampicillin resistant LB-Agar plates to form single colonies. Each representative colony for each specific GST-EZH2 fragment was then grown overnight in LB broth with 100ug/ml ampicillin at 37C and at 250rpm. The overnight culture was then diluted into LB broth the next day at a 1:50 dilution factor and grown at 37C until an OD .300-.600 was obtained, where then 0.1mM IPTG was added for 4hrs at 37C, all at 250rpm. Culture flasks were then moved to room temperature incubation and shook vigorously until pellets were harvested the next day by centrifugation at 6000rpm. Pellets were lysed in 20% sarkosly detergent solution, sonicated until viscosity reduced, and GST protein was extracted from the supernatant by incubation with GST-sepharose beads overnight at 4C. GST-EZH2 proteins were eluted from the sepharose beads using 50mMM reduced glutationine solution pH 8.0. Eluent was gel-filtered, used size appropriate gel filtration centrifugation columns from Millipore.

3.7.4. *in vitro* kinase assay

GST proteins (i.e.GST-EZH2 variants) were isolated and purified as described in 3.7.3. Buffer exchange was performed to store eluted GST proteins in 20mM Tris-HCl PH 7.4. Approximately 1ug of eluted GST protein was mixed with 0.2ul purified cyclin E/CDK2 kinase complex purchased from Millipore in kinase buffer (60 mM HEPES at pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 3 μM Na_3VO_4 and 1.25 mM DTT) in the presence of 1 μCi γ^{32}P ATP at 37°C for 30 min. SDS-DTT protein sample buffer was then added to the reaction and samples were denatured for 10min at 100°C. Samples were then analyzed by SDS-PAGE, stained with coomassie to determine protein loading, and incubated with film for overnight or for appropriate time at -80°C to determine phosphorylation levels.

3.7.5. *in vitro* S^{35} labeling and pull-down

Recombinant GST-EZH2 proteins prepared as described in 3.7.4 were incubated with the in-vitro transcribed and translated product HA-CDK2, which was produced using a TNT coupled reticulocyte lysate system from Promega. Incubation occurred in binding buffer for 1hr at 4°C. Rabbit IgG antibody or anti-HA antibody was then added after incubation at 4°C for overnight immunocomplexing. The product from the pull-down assay was washed extensively with binding buffer or PBS, and the bound proteins were eluted with SDS-DTT protein sample buffer for later SDS-PAGE analysis.

3.8. Viral shRNA infection

3.8.1. Production of lentiviral particles

Plko.1 vector based shRNA constructs were purchased from Acdemic Sinica, Taiwan. Respective EZH2 shRNA constructs were designated shEZH2 #3 and shEZH2 #4 (3'-UTR). Respective CDK2 shRNA constructs were designated shCDK2 #587 and shCDK2 #590 (3'-UTR). The control knockdown construct used to ensure any non-specific or off-target knockdown was a luciferase shRNA designated shLuc. To form lentivirus particles VSV-G, ΔR89.1, the respective plko.1 shRNA plasmids were transfected to $5.0x10^5$ 293T cells, seeded in 10cm dishes 12hr before transfection, as previously mentioned in methods section 3.1. The plasmid transfection ratio was 6:4:1, respectively. 6hrs after transfection, opti-medium was changed to 1% BSA containing 10% medium. Viral supernatants were then concentrated using lentiviral concentrator-X from Clonetech after centrifugation, aliquoted, and stored at -80C.

3.8.2. Virus infection and polyclonal stable line selection

Prior to virus infection, target cells were seeded based on their growth rate in 6 well dishes, roughly to a cell number of $2.0\ x10^5$ cells. Target cells were then incubated with 100ul of aliquots described in 3.8.1. and 1.5ml-2.0ml of 10% medium after 1hr centrifugation at 2500rpm. Each infection reaction was supplemented with 8ug/ml polybrene/DMSO from Millipore. Cells were incubated for 24hrs and medium was changed to 10% FBS medium or to a second viral transduction. After primary infection, puromycin was added to a concentration of 1ug/ml at approximately 72hrs for selection of polyclonal cell populations.

Puromycin selection occurred for 1 week and puromycin supplementation was replace every 3 days. Polyclonal stable lines were then analyzed by SDS-PAGE and cryopreserved in liquid nitrogen for future experiments.

3.9. Immunohistochemical (IHC) staining

IHC staining was performed using a immunoperoxidase-based staining method via a AEC detection kit from Pierce. Tissue microarray (TMA) slides were prepared from the respective breast cancer patient paraffin-embedded cohort 5uM sections. Paraffin embedded sections were deparaffinized in xylene followed by serial rehydration using ethanol dilution gradients. Antigen recovery was achieved by microwaving samples for 8min in 10 M sodium citrate buffer PH 6.0 followed by room temperature incubation for 30min and 2X was in PBS. The slides were then trypsinized using 0.05% trypsin in PBS at room temperature for 15min followed by 3X wash in PBS (each wash being 5min). Endogenous or basal level peroxidase activity was reduced by an addition of 0.3% H_2O_2 in methanol for an incubation period of 15min at room temperature followed again by 3X wash in PBS (each wash being 5min). The slides were then blocked with 10% serum corresponding to the species of the primary antibody in PBS in a humid chamber at room temperature for 30min. The residual serum was blotted with chem-wipes. The primary antibody was applied to the slides and incubated overnight at 4C. Slides were washed the next day 3X in PBS then incubated with biotin-conjugated secondary anti-mouse or rabbit IgG for 1hr at room temperature. The avidin-biotin-horseradish peroxidase complex from Vector Labs was applied after being washed 3X in PBS. The avidin-biotin-horseradish

peroxidase complex was incubated for 1hr at room temperature followed by 3X PBS wash. Antibody signals were then detected by -ethyl carbazole chromogen kit (AEC) chromogen substrate kit from Pierce. The slides were counterstained with Mayer's hematoxylin and mounted in aqueous mounting from Lerner Laboratories. Finished slides were then visualized and evaluated under microscope by both lab pathologists and processed for final statistical analysis.

3.10. Chromatin immunoprecipitation (ChIP) assay

Cells were fixed with 5% formaldehyde for 10 min, washed, and lysed in cell lysis buffer (5 mM HEPES [pH 8.0], 85 mM KCl, 0.5% NP-40) for 30min at 4C. Fixation was stopped using 5M glycine addition. Cells were then lysed in modified RIPA buffer and the lysate sonicated on ice to achieve DNA fragmentation of approximated 500-1000bp. The supernatant was diluted 10-fold with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 6.8, and 167 mM NaCl). Chromatin immunoprecipitation was achieved by adding 5µg of target antibody to 1mg of protein lysate overnight at 4C. Protein/DNA immunocomplexes were then pulled down by protein A/G-conjugated agarose beads. The beads were washed with wash buffer (0.1 M sodium phosphate buffer PH 6.8, 0.1% Tween-20) for 3X, and the bound protein was eluted twice with 30 µl 0.1 M citrate PH 3.0 and pooled. Extraction buffer (0.1% SDS, 50 mM $NaHCO_3$, 5 µl of 10 mg/ml RNase, 18 µl of 5 M NaCl) was added to elute the protein from the DNA and incubated at 65°C overnight to digest residual RNA. The retrieved DNA was purified with a Qiagen miniprep spin

column and eluted in water. The promoter regions were amplified by conventional PCR or qRT-PCR.

3.11. Real time RT-PCR

RNA was extracted from cells of interest using a Qiagen RNeasy kit. The first strand synthesis was performed to convert RNA to cDNA using Bio-Rad first strand synthesis kit and qRT-PCR was performed as previously described. The gene expression was compared relatively to 18s rRNA.

3.12. Colony formation assay

Cells were seeded in ranges of 500, 1000, and 5000 cells per well in a 6 well dish and grown for 10 days in 10% FBS medium. Colony formation was assessed via fixation, crystal violet staining for 5min, followed by overnight wash in PBS or water. Colonies were counted under microscope for quantitation.

3.13. MTT cell proliferation assay

Cells were seeded at $1.0X10^4$ and grown in 10% FBS medium. Each day MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to cells and incubated for 4-6hrs. The insoluble formazan salt was dissolved using DMSO the optical density and was determined at 570nm.

3.14. Soft-agar anchorage independent growth assay

50,000 cells were seeded in 12 well plates in top layer 0.25% agarose on top of base layer of agarose composition of 0.5% agarose. Agarose layers were both supplement to 10% FBS medium. Cells were grown for 6-8

weeks with every second-day fresh 10% medium being added to the dish. After 8 weeks visible colonies were stained with crystal violet for 5min and washed overnight with PBS. Colonies were counted and visualized under light microscope for quantitation.

3.15. Migration assay

Migration was measured in 24-well migration chamber plates with a 8μm pore size polycarbonate filter purchased from BD Biosciences. The lower chamber contained 0.75ml of 10% FBS medium. The upper chamber contained $1.0X10^4$ cells seeded in serum-free medium. Cell migration was carried out for 24hrs in cell incubator. After migration, the filter was fixed with 4% glutaraldehyde in PBS and stained with crystal violet. Unmigrated cells were removed from on top of the filter with a cotton swab. Migrated cells were then counted under microscope for quantitation after destain.

3.16. Invasion assay

Invasion was measured in 24-well migration chamber plates with a 8μm pore size polycarbonate filter coated with growth factor free matrigel purchased from BD Biosciences. The lower chamber contained 0.75ml of 10% FBS medium. The upper chamber contained $5.0X10^4$-$1.0X10^5$ cells seeded in serum-free medium. Cell migration was carried out for 24hrs in cell incubator. After migration, the filter was fixed with 4% glutaraldehyde in PBS and stained with crystal violet. Unmigrated cells were removed from on top of the filter with a cotton swab. Migrated cells were then counted under microscope for quantitation after destain.

3.17. Anoikis Assay

6 well-tissue culture dishes were pre-coated with poly-HEMA concentration of 5mg/ml at 37C and was 3X with PBS before seeding cells. $5.0X10^5$ cells were suspended in each well in culture medium containing 0.5% methylcellulose. Cells were stained with ethidium bromide homodimer and calcein-AM, both from molecular probes for 30min at 37°C to determine apoptotic and viable cell populations, respectively. Fluorescent signal was detected and quantified under microscope.

3.18. Mammosphere assay

Breast cancer cells were grown to confluence of 80-90% in a 2-D monolayer in 10% FBS medium. Cells were then trypsinized, washed 3X in PBS, and suspended in Stem Cell Technology, Inc. complete Mammocult medium for later seeding in non-adherent 12-well culture dishes at seeding density of $1.0X10^4$ per well. Sphere formation was then assessed at day 7 or day 10. Inhibitor addition was performed after normalizing sphere seeding to 100 spheres for each well treated with inhibitor.

3.19. 3-D growth assay

Cells were grown to 80-90% confluence, trypsinized, washed in PBS, and harvested or suspended in harvesting buffer from Trevigen. Cells were then seeded to 3.0X103 per well, with addition of 3-D Culture Cell Proliferation Reagent to each well according to Trevigen assay protocol. Cells were then

incubated at 37°C, 5% CO2. Inhibitors were added to spheres at day 2 at assessed concentrations from mammosphere assay.

3.20. Xenograft mouse model

Tumorigenesis assays were performed in an orthotopic breast cancer mouse model. MDA-MB 231 cells (2.0×10^6) with lentiviral-stable expression of vector, $EZH2^{WT}$, $EZH2^{T416A}$, $EZH2^{T416D}$, shLuc, shCDK2 #587, or shEZH2 #4 were injected into mammary fat pads of nude mice (n = 5 per group). Tumor size was measured every 3 days with a caliper and tumor volume was determined using the formula: $L \times W^2 \times 0.52$, where L is the longest diameter and W is the shortest diameter.

3.21. Statistical analysis

SAS software (version 8.1) was used for statistical analysis (SAS Institute). A univariate analysis was used to determine the variable distributions. Categorical variables among the groups were compared with the χ-squared test or Fischer's exact test if 20% of the expected values were smaller than five. Continuous variables were analyzed using the Student's t-test. A p-value of <0.05 was considered statistically significant.

.

CHAPTER 4. RESULTS

4.1 Defining the clinical significance of EZH2 and Cyclin E co-expression

The current clinical understanding in breast cancer oncology regarding basal-like breast cancer (BLBC) tumors and triple-negative breast cancer (TNBC) tumors is that they share a similarity determined by gene expression analysis and key biomarker expression. Overall 80% of BLBC are considered to be triple-negative (TN) in status based on IHC negative staining for estrogen-alpha and progesterone receptor, and lack of the HER2 gene amplification defined either by IHC or FISH. Previous reports demonstrate that EZH2 and Cyclin E/CDK2 both independently have been linked to the basal-like breast cancer phenotype, but no reported link between EZH2 and cyclin E/CDK2 has been reported in the literature. To begin to investigate the potential causal relationship between EZH2 and cyclin E/CDK2 in sporadic triple-negative breast cancers, the clinical correlation between the expression levels of EZH2 and cyclin E/CDK2 was examined in breast cancer patient cohorts of triple-negative and non-triple-negative groups.

4.1.1. Correlation analysis of patients of triple negative group

A tissue microarray TNBC tissue cohort of 122 patients was stained via immunohistochemistry for EZH2 and cyclin E. Cyclin E expression level (59%) was slightly larger than the low expression population (41%) as previously reported. Interestingly EZH2 expression levels closely correlated with cyclin E

($p<0.0001$) as illustrated in Table 4. EZH2 and cyclin E association is demonstrated by a representative IHC picture in Figure 4.

4.1.2. Correlation analysis of patients of the non-triple negative group

As previously reported the non-triple negative patient cohort consisting of 125 tissues demonstrated a population with less cyclin E expression (12%). EZH2 and cyclin E correlation was not observed with statistical significance in this cohort ($P=0.53$). The statistic result is shown in Table 4.

Table 4. Correlation between Cyclin E and EZH2.

A. IHC staining result of triple-negative breast cancer tissues

B. IHC staining result of non-triple negative breast cancer tissues

Table 4.

A. Relationships between EZH2 and Cyclin E expression in triple negative breast cancer patients

		EZH2 Expression			
		Negative	Positive	Total	*p-value*
Cyclin E	Negative	37 (30.3%)	13 (10.7%)	50 (41%)	
	Positive	30 (24.6%)	42 (34.4%)	72 (59%)	
Total		67 (54.9%)	55 (45.1%)	122 (100%)	p<0.0001

B. Relationships between EZH2 and Cyclin E expression in non-triple negative breast cancer patients

		EZH2 Expression			
		Negative	Positive	Total	*p-value*
Cyclin E	Negative	46 (36.8%)	64 (51.2%)	110 (88%)	
	Positive	5 (4%)	10 (8%)	15 (12%)	
Total		51(40.8%)	74 (59.2%)	125 (100%)	p=0.53

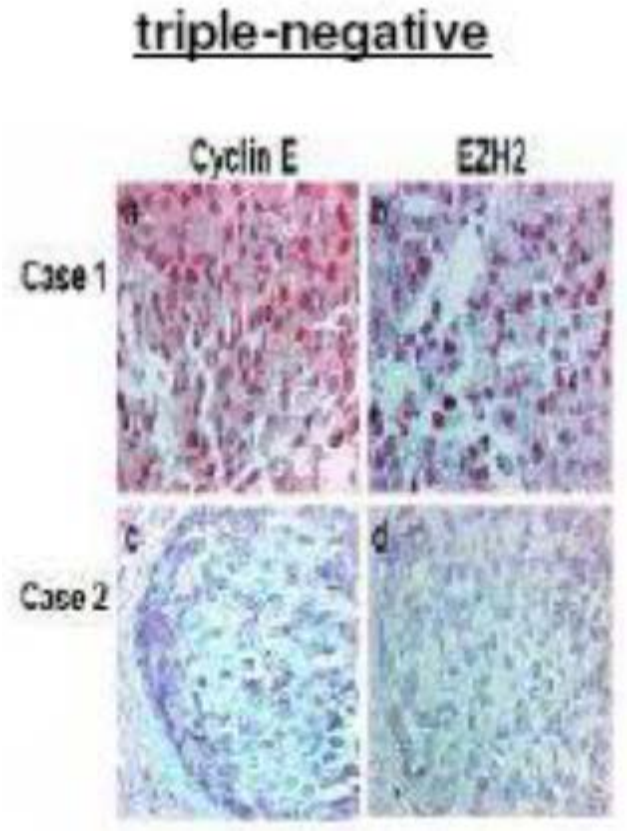
Figure 4. Case representation demonstrating association between expression of EZH2 and Cyclin E in TNBC and non-TNBC

A. Case representation of EZH2 and Cyclin E IHC in triple-negative breast cancer patient biopsy tissue

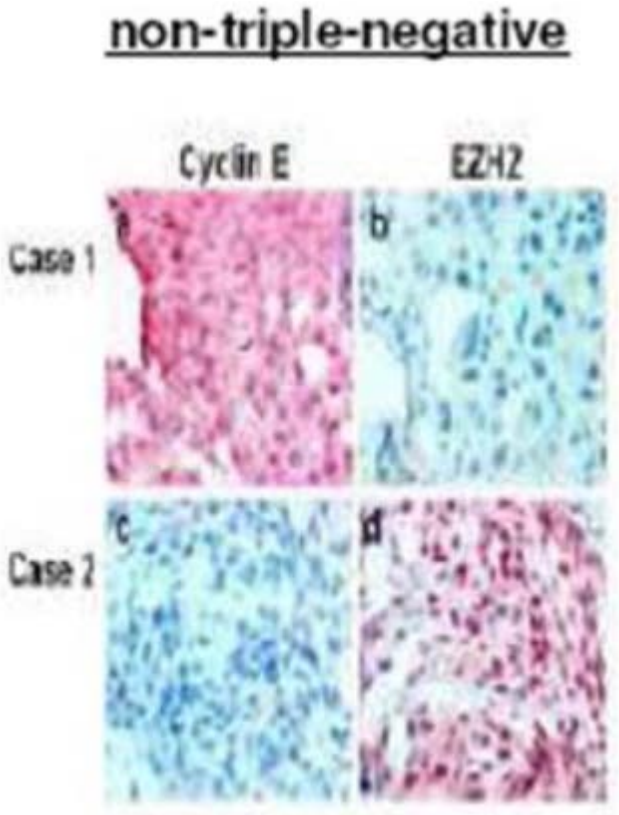
B. Case representation of EZH2 and Cyclin E IHC in non-triple-negative breast cancer patient biopsy tissue

Figure 4. Case representation demonstrating association between expression of EZH2 and Cyclin E in TNBC and non-TNBC

A



B



4.2. Physical association between EZH2 and cyclin E/Cdk2 complex

Cyclin E has many characterized cellular functions involved in many central dogmas of cancer. Many of these functions are orchestrated through CDK2, the only defined compliment enzymatic partner of cyclin E. CDK2 functions as a kinase capable of phosphorylating specific substrates causing changes in downstream signaling in normal and cancer cells. Knowing previously of the clinical correlation our group establish between cyclin E and EZH2, we pursued a potential regulatory mechanism of EZH2 being a phosphorylation substrate of CDK2. The physical association of EZH2 and CDK2 was examined both at a cellular level and *in vitro*.

4.2.1. Ectopic Co-IP in co-transfected 293T cells

The physical association was determined by co-transfection of 3X-Myc-EZH2 and HA-CDK2 in 293T cells at a 1:1 ratio followed by cell lysis and reciprocal Co-IP using either Myc or HA antibody.

4.2.2. Endogenous Co-IP in candidate triple-negative breast cancer cell lines

To confirm that the binding observed in the 293T system also occurs endogenously specific to TNBC cells, a panel consisting of TNBC cell lines MDA-MB 231, HS-578T, and BT-549 were immunoprecipitated using endogenous EZH2 antibody and later immunoblotted for CDK2.

4.2.3. Domain mapping of in vitro translated GST-EZH2 fragments and CDK2

In order to identify the EZH2 domain that CDK2 physically associates with HA-CDK2 was *in vitro* translated and labeled with S^{35} which was then incubated with GST-EZH2 fragments corresponding to domains I, II, and III (SET domain). EZH2 fragments corresponding to domains I, II, and III (SET domain) were pulled down using GST-sepharose beads and analyzed by SDS-PAGE and subsequent autoradiography. CDK2 was shown most significantly to associate with domain II of EZH2.

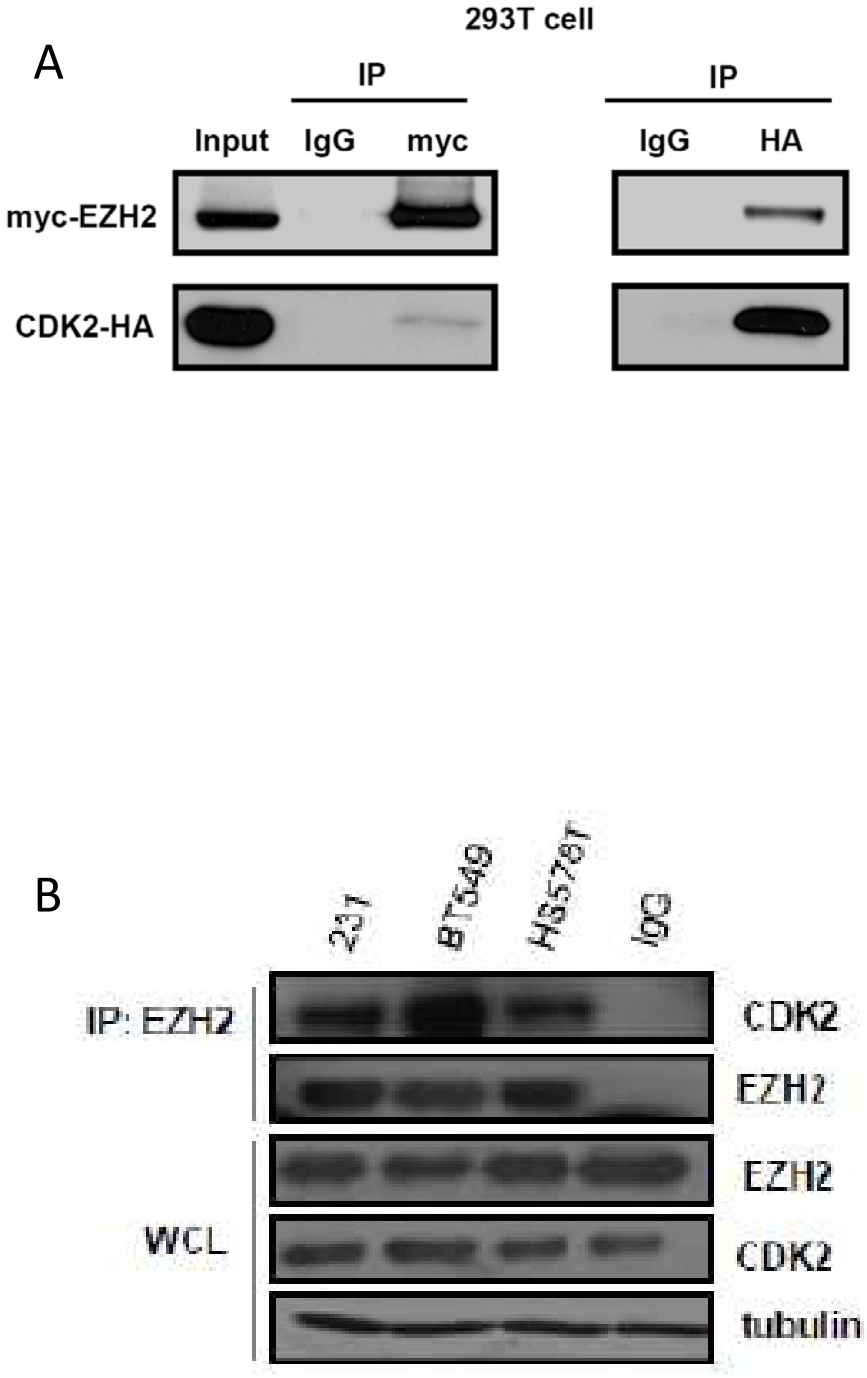
Figure 5. Cyclin E/CDK2 physically associates with EZH2

A. Lysates from 293T transfected with 3X-Myc-EZH2^{WT} and HA-CDK2 were immunoprecipitated using anti-MYC antibody and immunoblotted with anti-HA. Reciprocal experiments were done with corresponding antibodies

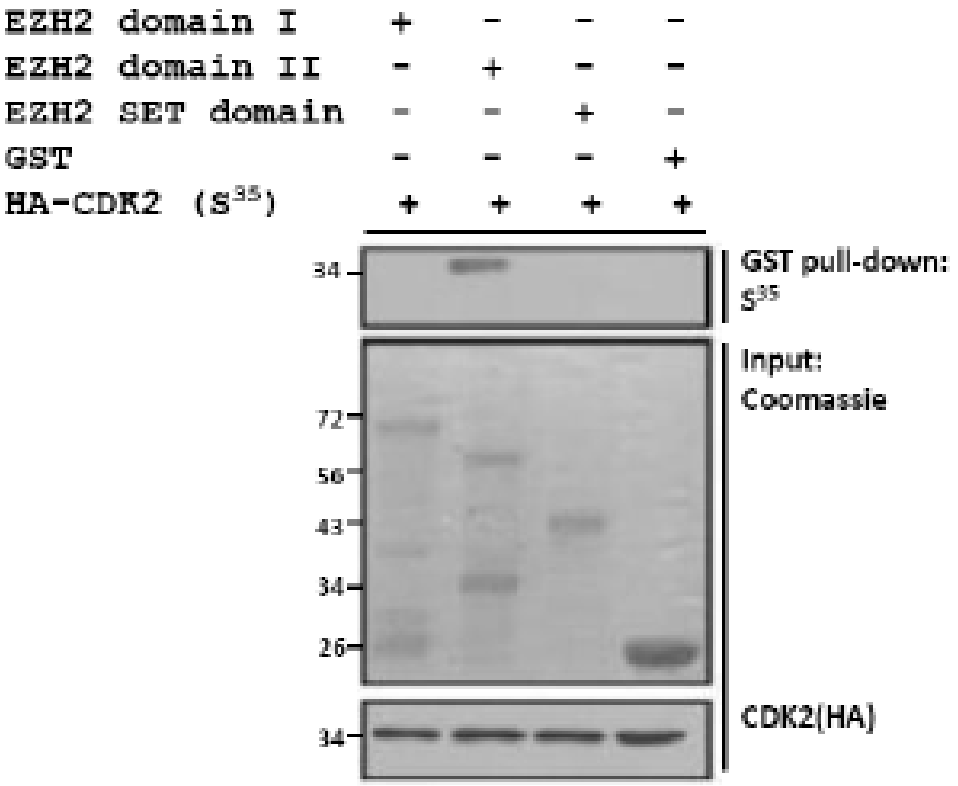
B. Triple-negative breast cancer lines MDA-MB 231, BT549, and HS578T cells were subjected to immunoprecipitation with anti-EZH2 antibody and immunoblotted with anti-CDK2 antibody.

C. HA-CDK2 was translated using *in vitro* translation assay, labeled with ^{35}S and incubated with GST-EZH2$^{1-333}$, GST-EZH2$^{334-610}$, or GST-EZH2$^{611-746}$. GST pull down mapped the CDK2 interaction domain within EZH2

Figure 5.


A
293T cell
IP
IP
Input
IgG
myc
IgG
HA
myc-EZH2
CDK2-HA
B
231
BT549
HS578T
IgG
IP: EZH2
CDK2
EZH2
WCL
EZH2
CDK2
tubulin

C


EZH2 domain I + - - -
EZH2 domain II - + - -
EZH2 SET domain - - + -
GST - - - +
HA-CDK2 (S35) + + + +
34
72
56
43
34
26
34
GST pull-down: S35
Input: Coomassie
CDK2(HA)

4.3. Cyclin E/CDK2 enhances phosphorylation of EZH2 on T416

CDK2 phosphorylation substrates commonly contain the consensus phosphorylation motif of (S/T)PX(K/H/R), where phosphorylation is directed by proline to either the serine (S) or threonine (T) residue adjacent. Mapping of the EZH2 phosphorylation was performed *in vitro* and was identified to significantly occur within domain II of EZH2 on T416, an evolutionarily highly conserved consensus CDK2 phosphorylation site motif (described in detail in 4.3.2.). Moving forward a monoclonal mouse antibody was generated recognizing endogenous phospho-T416 EZH2 through collaboration with our sister laboratory's antibody core facility, in Taiwan.

4.3.1. Proline-directed threonine phosphorylation of EZH2 is enhanced by Cyclin/CDK2

Cyclin E overexpression was observed to increase proline-directed threonine phosphorylation under co-transfection with 3X-Myc-EZH2. Threonine phosphorylation was mitigated through additional transfection of dominant negative HA-CDK2 or drug treatment with CDK2 inhibitors II, III, or Roscovitine suggesting threonine phosphorylation can be promoted by CDK2 kinase activity.

.

4.3.2. *in vitro* Cyclin E/CDK2 kinase mapping assay of GST-EZH2 fragments

Knowing threonine phosphorylation can be enhanced via a CDK2 kinase-activity-dependent manner we then mapped the phosphorylation residue using an *in vitro* cyclin E/CDK2 kinase mapping assay. To identify the exact threonine residue(s), first we generated and purified three GST-fused protein fragments of EZH2 were generated based on domain structure corresponding to domain I (1-333), domain II (334-610), and domain III or SET domain (611-746). GST-EZH2 fragments I, II and III were incubated with recombinant C-terminal 6X-His tag CDK2 and N-terminal GST tag Cyclin E in the presence of γP32-ATP in CDK2 kinase buffer (NEB), analyzed by SDS-PAGE, and developed using autoradiography. Uniquely EZH2 domain II demonstrated the most significant phosphorylation signal which coincided with the presence in domain II of a highly evolutionarily conserved threonine-CDK2 phosphorylation consensus motif. Site-directed point mutagenesis of T416A mitigated this phosphorylation signal, confirming T416 within domain II of EZH2 to be a CDK2 phosphorylation site.

4.3.3. *in vitro* Cyclin E/CDK2 kinase assay of full length GST-EZH2

To confirm the *in vitro* mapping assay identification of the T416 phosphorylation site, GST-EZH2 full length protein was purified and incubated with recombinant C-terminal 6X-His tag CDK2 and N-terminal GST tag Cyclin E in the presence of γP32-ATP, analyzed by SDS-PAGE, and developed using autoradiography. Site-directed point mutagenesis of T416A again mitigated this

phosphorylation signal yielding confirmation that this phosphorylation site also occurs in a full length full-length intact EZH2-GST fusion protein.

4.3.4. Generation of mouse polyclonal and monoclonal antibody targeting phospho-T416 EZH2

Mouse polyclonal antibody was generated by immunizing mice to a number of three to five times with a phospho-peptide (pT416) flanked by bilateral extension to the N-and C-terminal representative of the region encompassing T416, ANSRCQ-pT^{416}-PIKMK-MAP. Serums were screened for using a pT416-peptide based ELISA and positive serums were further verified by dot blot using competitive peptide titrations. For *in vivo* verification, co-transfection assays used in 4.3.1 were performed, using the phospho-mitigated EZH2 mutant T416A as a negative control. Moue monoclonal antibody (mAb) was screened for in several hybridoma cell lines using the ELISA method mentioned above. Confirmation of the specificity of the antibody for immunoblotting and IHC was also determined in a similar manner.

Figure 6. Identification and verification of EZH2 phosphorylation site by Cyclin E/CDK2

A. Lysates from 293T transfected with Cyclin E, Myc-EZH2^{WT}were immunoprecipitated using anti-MYC antibody and immunoblotted with anti-Myc, p-NPM, NPM, or anti-phospho-Threonine/proline antibodies. 293T cells were treated with and treated with CDK2 inhibitor I, II, or Roscovitine.

B. Schematic of GST-EZH2 fragment domain structure with hi-lite of the evolutionarily conserved CDK2 consensus phosphorylation motif.

C. Mapping of T416 EZH2 phosphorylation to GST-EZH2 protein fragment domain II using *in vitro* kinase assay

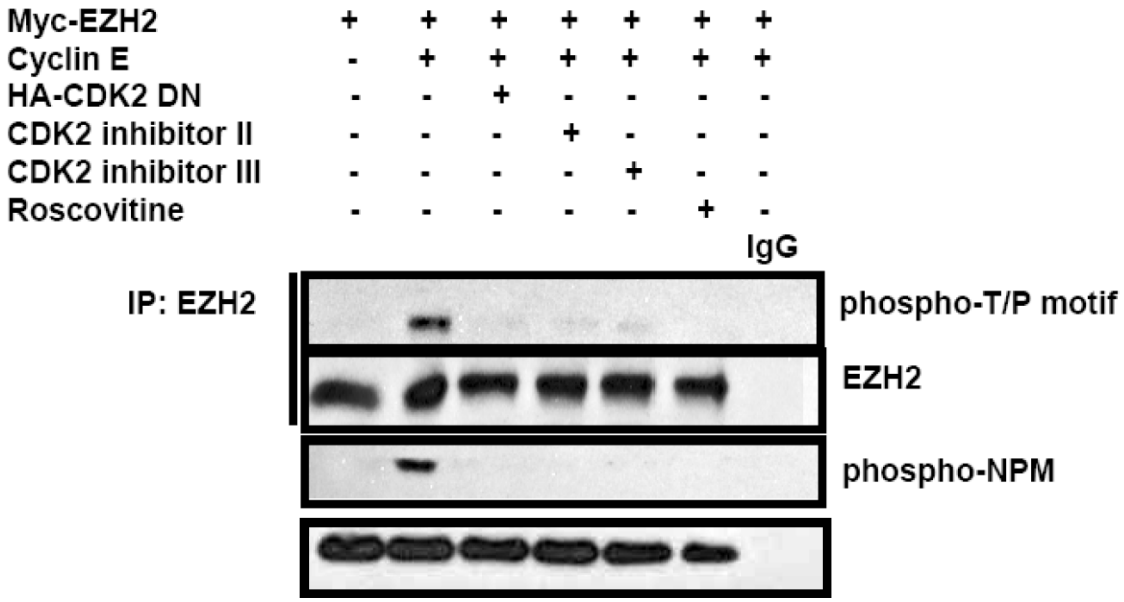
D. Identification and confirmation of T416 phosphorylation in full-length GST-EZH2 protein.

E**.** Lysates from 293T transfected with Cyclin E, Myc-EZH2^{WT} Myc-EZH2^{T416A} were immunoprecipitated using anti-MYC antibody and immunoblotted with anti-Myc, p-NPM, NPM, or anti-phospho-T416 EZH2 antibodies after immunoprecipitated using anti-MYC antibody. 293T cells were treated with and treated with CDK2 inhibitor I, II, or Roscovitine.

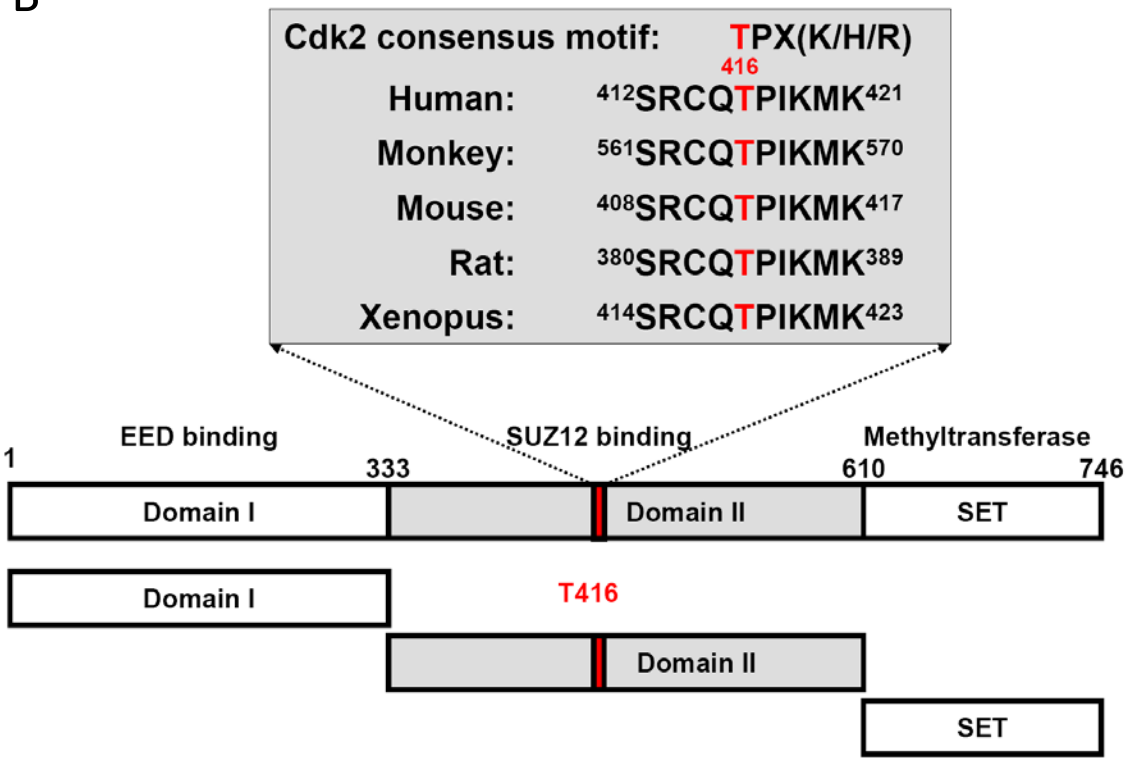
F. Dot blots characterization of phospho-T416 EZH2 antibody under phospho-peptide competition assay.

Figure 6.

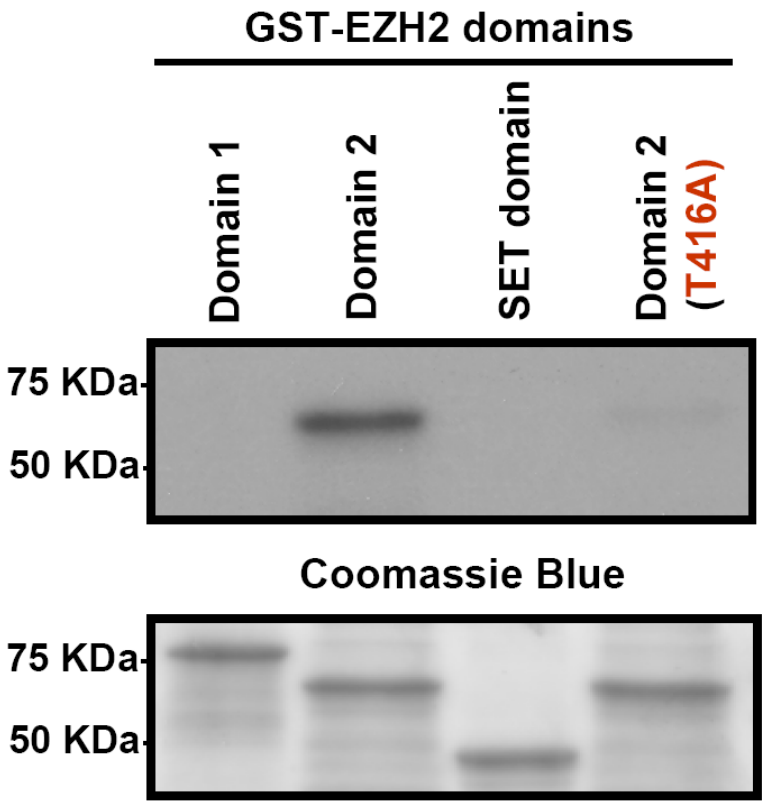
A


Myc-EZH2 + + + + + + +
Cyclin E - + + + + + +
HA-CDK2 DN - - + - - - -
CDK2 inhibitor II - - - + - - -
CDK2 inhibitor III - - - - + - -
Roscovitine - - - - - + -
IgG
IP: EZH2
phospho-T/P motif
EZH2
phospho-NPM

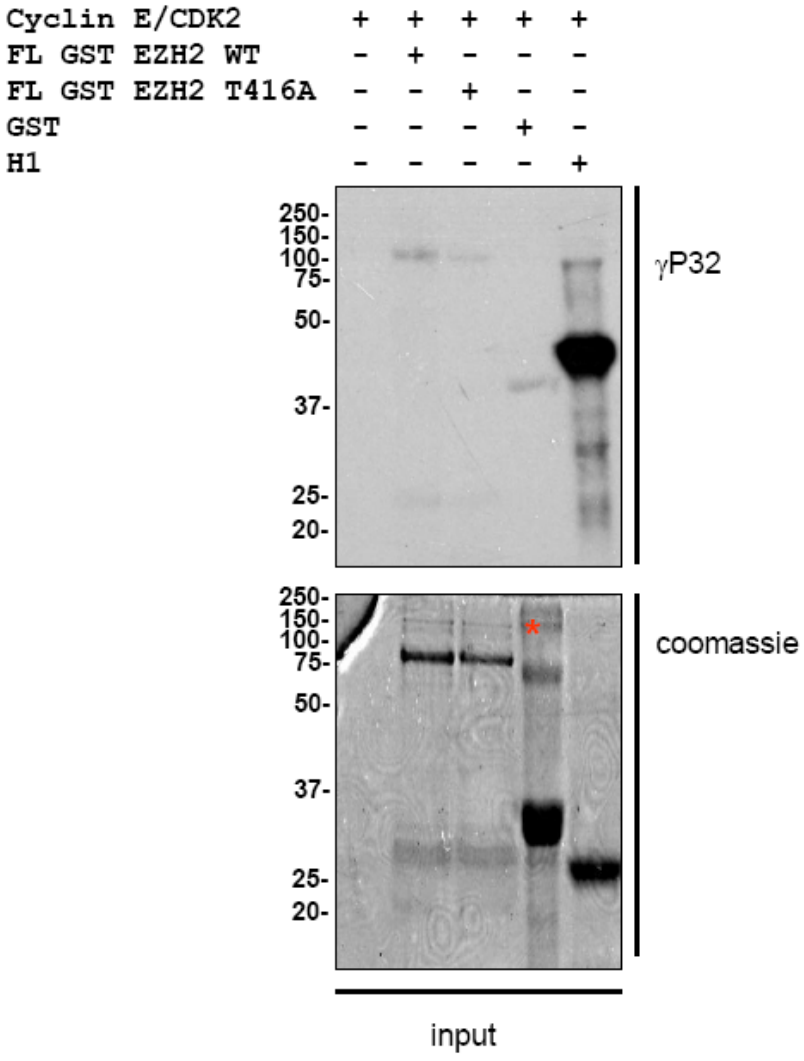
B


Cdk2 consensus motif: TPX(K/H/R)
416
Human: 412SRCQTPIKMK421
Monkey: 561SRCQTPIKMK570
Mouse: 408SRCQTPIKMK417
Rat: 380SRCQTPIKMK389
Xenopus: 414SRCQTPIKMK423
EED binding
SUZ12 binding
Methyltransferase
1
333
610
746
Domain I
Domain II
SET
Domain I
T416
Domain II
SET

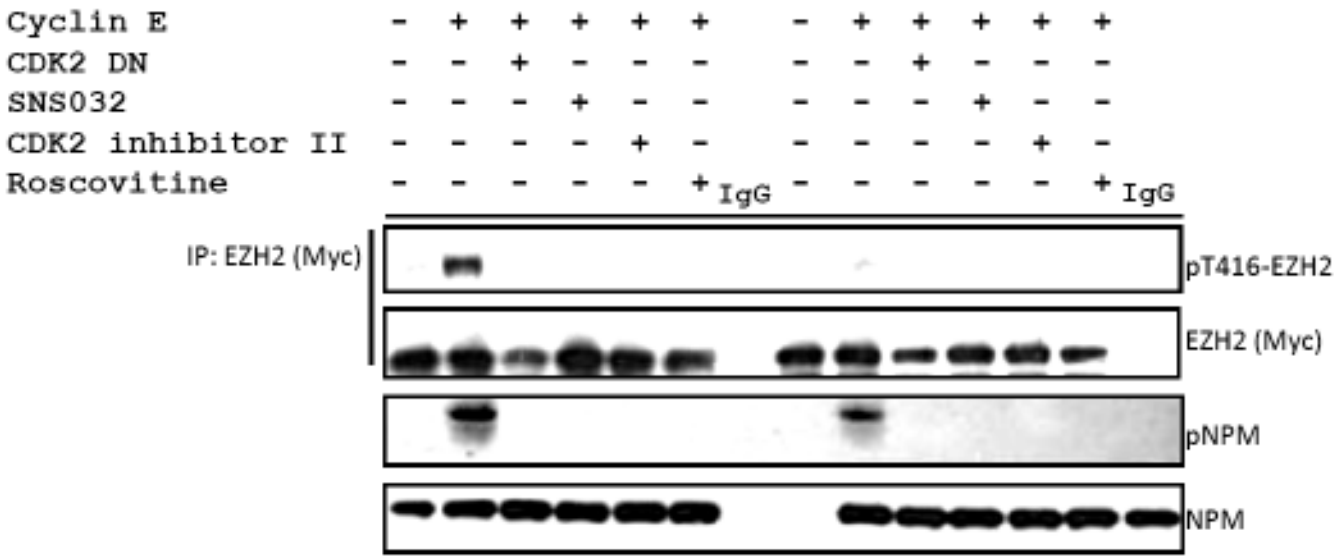
C


GST-EZH2 domains
Domain 1
Domain 2
SET domain
Domain 2 (T416A)
75 KDa
50 KDa
Coomassie Blue
75 KDa
50 KDa

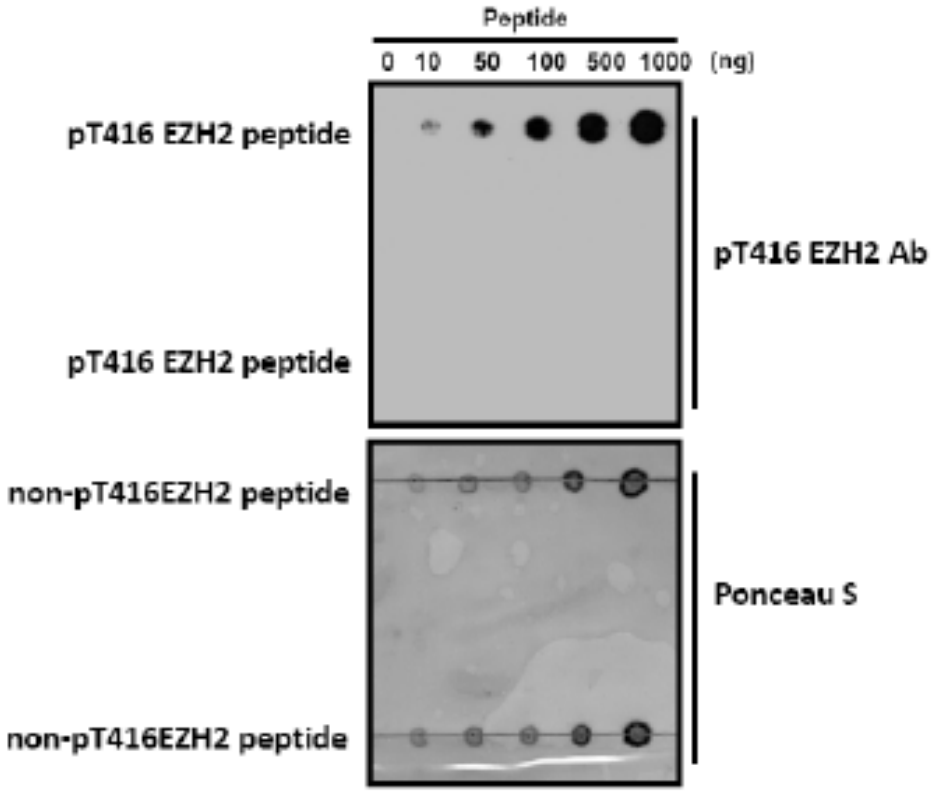
D


Cyclin E/CDK2 + + + + +
FL GST EZH2 WT - + - - -
FL GST EZH2 T416A - - + - -
GST - - - + -
H1 - - - - +
250-
150-
100-
75-
50-
37-
25-
20-
γP32
250-
150-
100-
75-
50-
37-
25-
20-
*
coomassie
input

E


Cyclin E - + + + + + - + + + + +
CDK2 DN - - + - - - - - + - - -
SNS032 - - - + - - - - - + - -
CDK2 inhibitor II - - - - + - - - - - + -
Roscovitine - - - - - + IgG - - - - - + IgG
IP: EZH2 (Myc)
pT416-EZH2
EZH2 (Myc)
pNPM
NPM

F


Peptide
0 10 50 100 500 1000 (ng)
pT416 EZH2 peptide
pT416 EZH2 peptide
pT416 EZH2 Ab
non-pT416EZH2 peptide
non-pT416EZH2 peptide
Ponceau S

4.4 T416 phosphorylation increases TNBC patient mortality

EZH2 phosphorylation on T416 serving as potential biomarker for BLBC/TNBC patients may function as a predictive tool to determine which BLBC/TNBC patients are at benefit for CDK2 or EZH2 inhibitor based therapies. Very few predictive tools are available for BLBC patients and T416 phosphorylation therefore could serve as one of the first in kind predictive therapy markers. Further validation of such mentioned CDK2 and EZH2 therapies are shown later on in the BLBC/TNBC cell line tumor sphere killing assay.

4.4.1 Survival analysis of TNBC patients exhibiting high levels of T416 phosphorylation

Generation of the monoclonal EZH2 T416 phosphorylation antibody permitted further investigation into the TNBC cohorts. Survival data was available for most of the 125 TNBC tissue samples originally used to establish the clinical correlation between Cyclin E and EZH2 co-expression. Out of the 125 tissue samples on the TNBC TMA 100 were available for staining and had corresponding survival data available. Using the mouse mAb for phospho-T416-EZH2 (pT416) we determined TNBC patients with high T416 phosphorylation had shorter survival time compared to those TNBC patients with low pT416. The specificity of the pT416-mAB was determined using a native and phospho-peptide competition staining control.

4.4.2 Survival analysis of non-TNBC patients exhibiting high levels of T416 phosphorylation

Survival data was available for a more limited number of the 125 non-TNBC tissue samples originally used to establish the clinical correlation between cyclin E and EZH2 co-expression. Out of the 125 tissue samples on the TNBC TMA, 79 were available for staining and had corresponding survival data available. Using the mAb for phospho-T416-EZH2 (pT416-EZH2) we determined there was not a correlation between pT416 levels and survival in non-TNBC patients.

Figure 7. Correlation between pT416 levels and patient survival in triple-negative and non-triple negative breast cancer

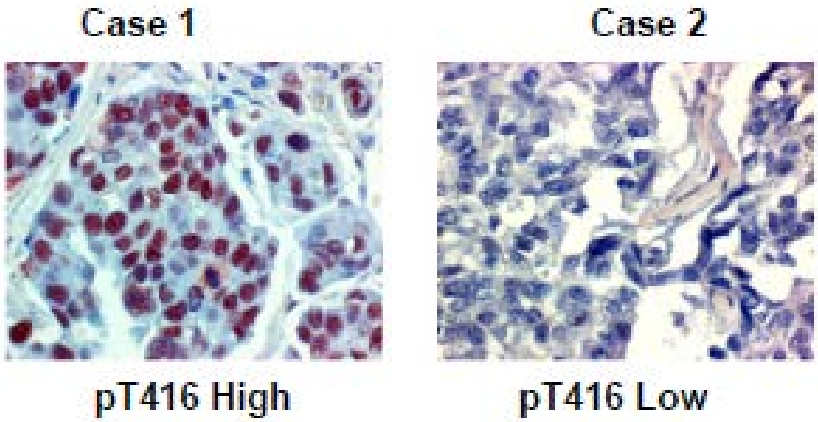
A. Case representation of high EZH2-T416 phosphorylation (pT416) and low EZH2-T416 phosphorylation

B. Survival analysis of patients with high and low pT416 in triple-negative breast.

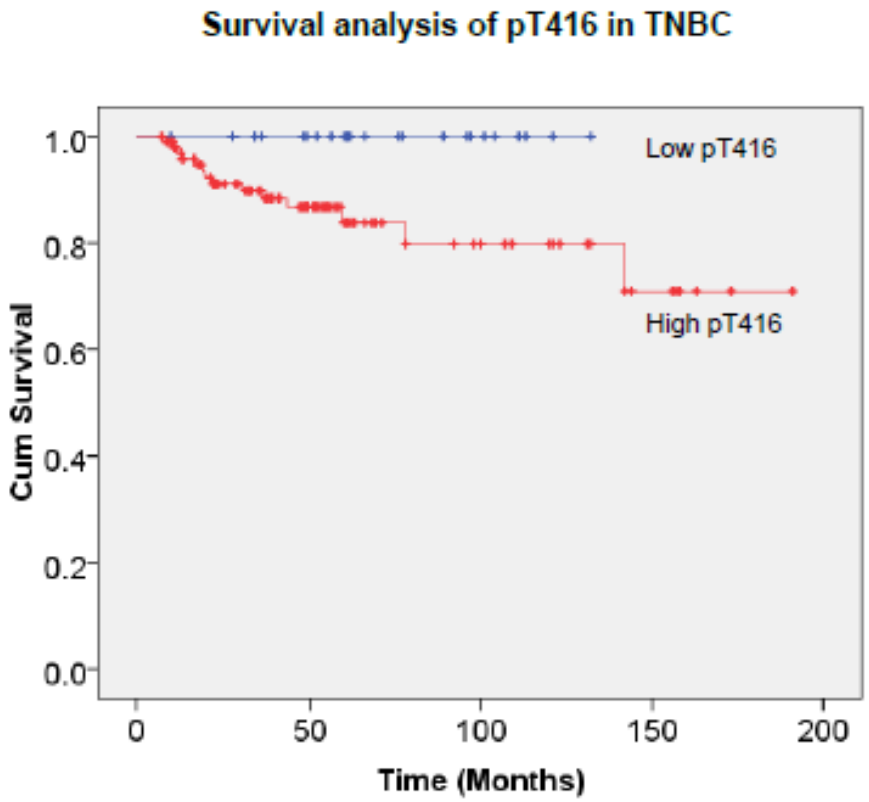
C. Survival analysis of patients with high and low pT416 in non-triple-negative breast.

Figure 7.

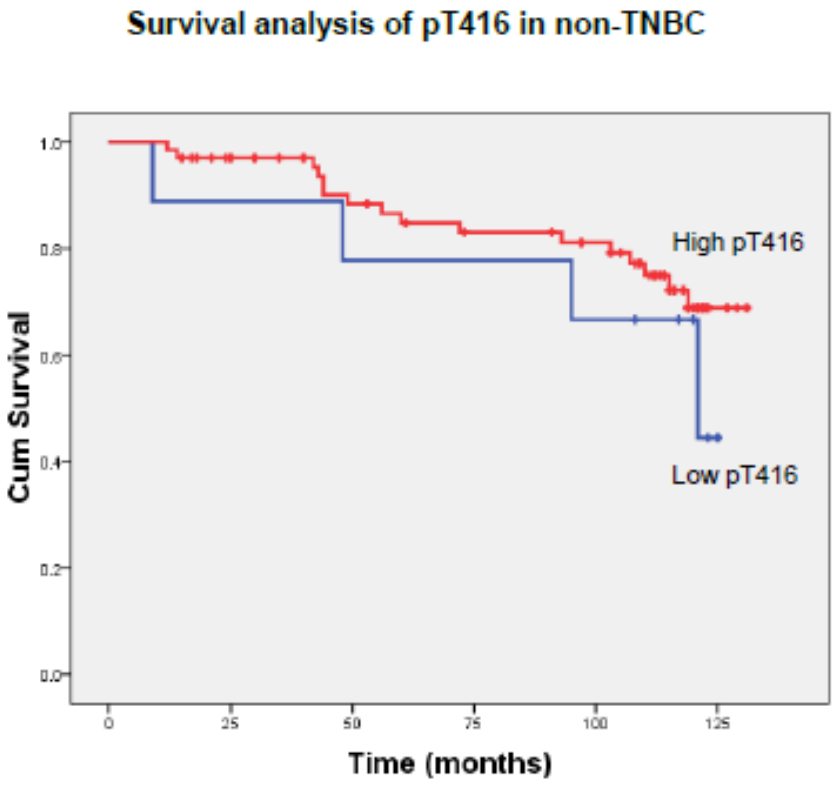
A


Case 1
Case 2
pT416 High
pT416 Low

B


Survival analysis of pT416 in TNBC
Low pT416
High pT416
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
Time (Months)

C


Survival analysis of pT416 in non-TNBC
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
25
50
75
100
125
Time (months)
High pT416
Low pT416

4.5 Cell cycle dependency of T416 phosphorylation

EZH2 is a cell cycle regulated protein transcriptionally controlled by the Rb/E2F transcriptional pathway at the beginning of G1/S phase of the cell cycle. CDKs are well known for controlling cell cycle checkpoints. Recently EZH2 has been shown to be phosphorylated by both CDK1/CDK2 on T345 and phosphorylated by specifically by CDK1 on T487. Functionally the T345 residue was capable of changing lncRNA binding with EZH2 while T487 phosphorylation disrupted the PRC2 complex association to inhibit mesenchymal stem cell differentiation. Both phosphorylation residues were shown to be cell cycle-dependent when observed under double thymidine or nocodazole synchronization conditions.

4.5.1 T416 phosphorylation is enhanced in G1/S stage of the cell cycle

The cell cycle dependency of the EZH2 T416 phosphorylation was determined by double thymidine block to synchronize cells in the G1/S phase to a level of %80 synchronization. Cells were then released from double thymidine blockade and the custom pT416 mAB was used to monitored T416 phosphorylation as cells progressed from G1/S to the G2/M stage of the cell cycle. In Hela cells, a canonical cell synchronization model cell line, and in the BLBC cell line, MDA-MB-231, T416 phosphorylation peaked at G1/S when the cyclin E level also was the highest. As cyclin E levels decreased in both cell lines the T416 phosphorylation also decreased and was diminished upon entry into G2/M. This shows T416 phosphorylation is enhanced through cyclin E expression during the cell cycle and can be sustained by cyclin A after G1/S to a

lesser extent until CDK2 activity is depleted upon entering M-phase. Upon entering M-phase it is predicted that the T416 phosphorylation is reduced to a greater extent when compared to G2/M, if not abolished completely.

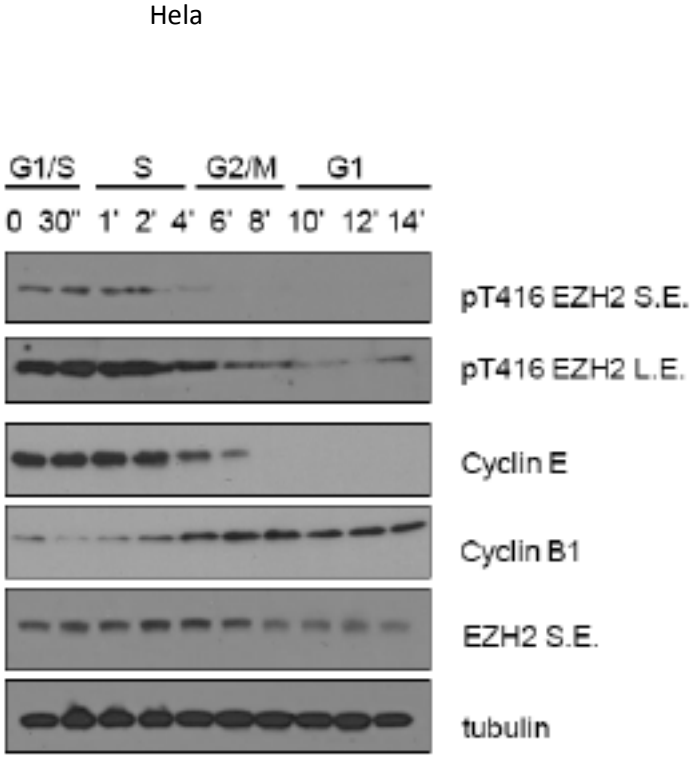
Figure 8. Cell cycle dependency of EZH2 T416 phosphorylation

A. Hela cells synchronized and released using double-thymidine block and monitored from 0, 30min (“) to 14hrs (‘) after removal of thymidine block

B. MDA-MB 231 cells synchronized and released using double-thymidine block and monitored from 0, 30min (“) to 14hrs (‘) after removal of thymidine block

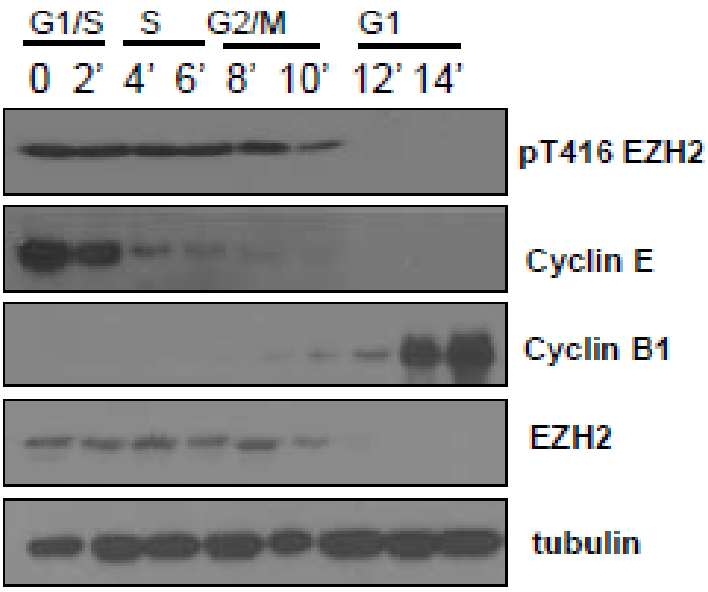
Figure 8.

A


Hela
G1/S
S
G2/M
G1
0 30" 1' 2' 4' 6' 8' 10' 12' 14'
pT416 EZH2 S.E.
pT416 EZH2 L.E.
Cyclin E
Cyclin B1
EZH2 S.E.
tubulin

B

MDA-MB 231


G1/S
S
G2/M
G1
0 2' 4' 6' 8' 10' 12' 14'
pT416 EZH2
Cyclin E
Cyclin B1
EZH2
tubulin

4.6 EZH2 T416 phosphorylation enahnces measures of breast cancer agressiveness similar to BLBC

EZH2 enhances many of the Hanahan and Weinberg described cancer cell dogmas. In order to determine if EZH2 T416 phosphorylation can increase breast cancer tumorigenesis similar to BLBC/TNBC we tested cellular functions paralleled by known oncogenic functions of EZH2 in promoting breast cancer aggressiveness similar if not overlapping with the aggressive traits intrinsic to BLBC/TNBC.

4.6.1 EZH2 T416 phosphorylation does not enhance 2-D growth of breast cancer

BLBC/TNBC breast cancer often has a higher proliferative rate as determined by ki67 antigen IHC staining in patient biopsies. Other growth assays have also been used to determine increases in breast cancer cell proliferation such MTT, Brdu, or CFU. In order to determine if T416 phosphorylation can enhance BLBC/TNBC cell proliferation MDA-MB 231-vector, MDA-MB 231-EZH2-WT, MDA-MB 231-EZH2-T416A and MDA-MB 231-EZH2-T416D stable clones were generated via lentiviral infection expressing the respective EZH2 point mutation variants. Proliferation rates amongst the MDA-MB 231 cells with EZH2 overexpression did not have a change in cell proliferation when measured by MTT or CFU assay suggestion T416 phosphorylation does not increase growth of BLBC/TNBC cells under 2-D culture conditions. CDK2 knockdown (MDA-MB 231 shCDK2) and EZH2 (MDA-MB 231 shEZH2) knockdown by shRNA lentiviral infection demonstrated reduction in cell growth measured by

both CFU and MTT assay confirming altering EZH2 or CDK2 functional levels through reducing protein levels was able to change growth of MDA-MB 231 cells in a 2-D culture environment. Because breast cancer cells often require a 3-D support to properly reflect their normal growth behavior in tissue culture settings we treated the 2-D growth result as an artifact of culture conditions and looked to confirm the result under 3-D growth conditions using the above mentioned cell lines cultured in a soft-agar anchorage-independent growth setting.

4.6.2 EZH2 T416 phosphorylation enahnces breast cancer cell growth in a 3-D dependent manner

As described MDA-MB 231-vector, MDA-MB 231-EZH2-WT, MDA-MB 231-EZH2-T416A, and MDA-MB 231-EZH2-T416D stable clones generated via lentiviral infection were cultured under 3D-growth conditions or formally known as soft-agar anchorage-independent growth conditions. Differences in growth characteristics were more readily observed. Under soft-agar growth conditions T416A expressing cells exhibited reduced colony formation capacity compared to WT expressing cells. Conversely, T416D expression increased the colony formation capacity of MDA-MB 231 cells compared to WT expressing cells. This data suggests that the colony formation capacity of MDA-MB 231 cells can be increased through expression of the T416 EZH2 phosphorylation phospho-mimetic point mutant expression, T416D, and reduced when the cells express the T416-phospho null EZH2 point mutant. Similarly to 2-D culture conditions EZH2 or CDK2 knockdown also reduced cell growth under soft agar conditions Overall this suggests modulation of T416 phosphorylation represented by alanine

(A) or aspartic acid (D) point mutation can reduce or enhance BLBC/TNBC cell growth in 3-D growth culture environment, respectively.

4.6.3 EZH2 T416 phosphorylation enhances breast cancer migration and invasion

EZH2 overexpression and phosphorylation by CDK1 on T487 have been shown influence breast cancer cell migration and invasion. Invasion and migration increase when EZH2 expression level is elevated in many normal and cancer cell types, including BLBC. We examined the stable clones mentioned in 4.6.1 that stably express EZH2-(WT, A, D) in the modulatory context of T416 phosphorylation previously mentioned. Similar to the growth advantage gained based on the represenative phosphorylation mimics by cells expressing EZH2 D-form form compared to EZH2 WT, these cells were also observed to have an increased ability in their migration through boden chamber pores and invasion ability through growth factor-reduced matrigel filters. EZH2 A-form expressing cell migration and invasion ability were mitigated compared to EZH2 WT expressing cells. Similarly to 2-D culture conditions EZH2 or CDK2 knockdown also reduced cell migration and invasion. This data suggests that EZH2 T416 phosphoryaltion may be able to provide BLBC cells with an ability to home to growth factor signaling and cross tissue barriers mimicing in an *in vitro* manner, metasastic cellular programs.

Figure 9. T416 phosphorylation enhances 3-D dependent growth and migration/invasion

A. Overexpression of vector control (V), and 3XMyc-EZH2-WT(WT), EZH2-T416A (A), EZH2-T416D (D) . Lower panel is lentiviral luciferase knockdown control (shLuc), EZH2 knockdown (shEZH2), and CDK2 knockdown (shCDK2). Both upper and lower panel are in MDA-MB 231 breast cancer cells.

B, C. Colony formation assay using lentiviral V and EZH2 -WT, A, D-forms shLuc, shEZH2 and shCDK2 MDA-MB 231 cells.

D, E. MTT assay using lentiviral V and EZH2 -WT, A, D-forms of shLuc, shEZH2 and shCDK2 MDA-MB 231 cells.

F, G. Soft-agar assay using lentiviral V and EZH2 -WT, A, D-forms shLuc, shEZH2 and shCDK2 MDA-MB 231 cells.

H. Migration assay using lentiviral V and EZH2 -WT, A, D-forms of MDA-MB 231 expression cells

I. Invasion assay using lentiviral V and EZH2 -WT, A, D-forms of MDA-MB 231 expression cells

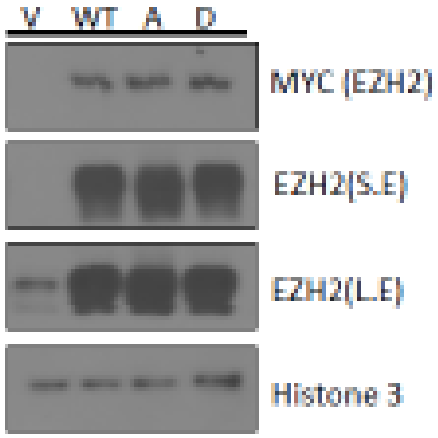
J. Migration assay using lentiviral shLuc, shEZH2 and shCDK2 MDA-MB 231 cells.

K. Invasion assay using lentiviral shLuc, shEZH2 and shCDK2 MDA-MB 231 cells.

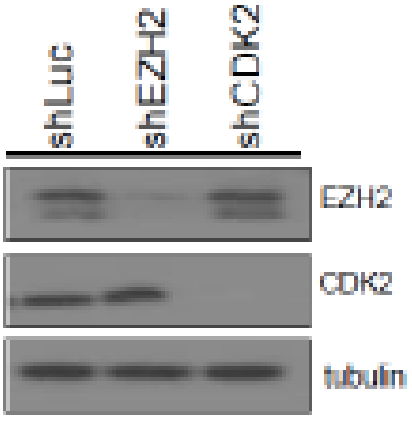
Figure 9.

MDA-MB 231

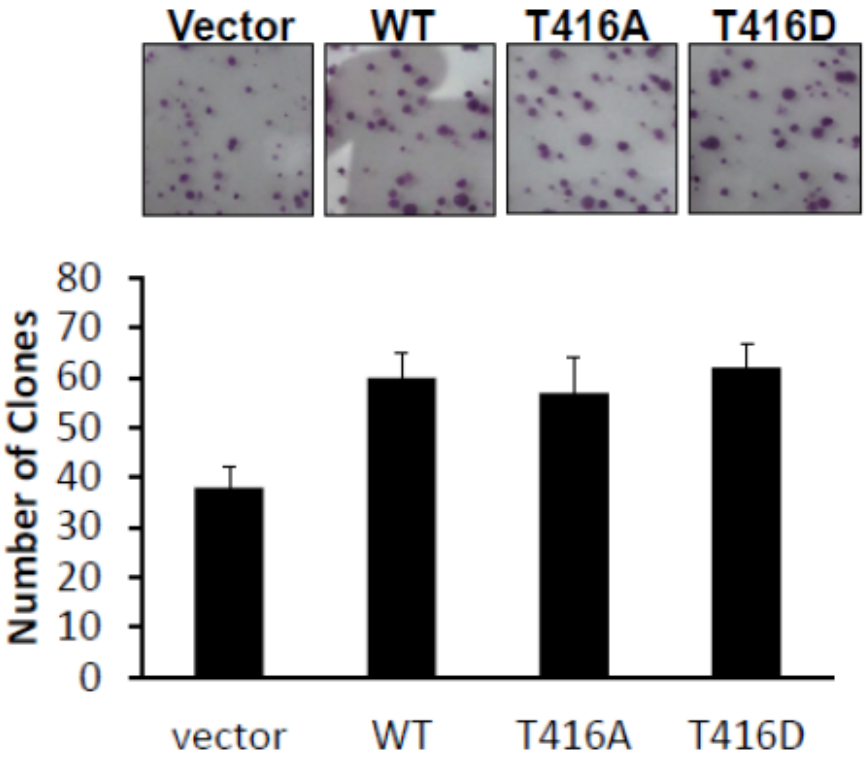
A



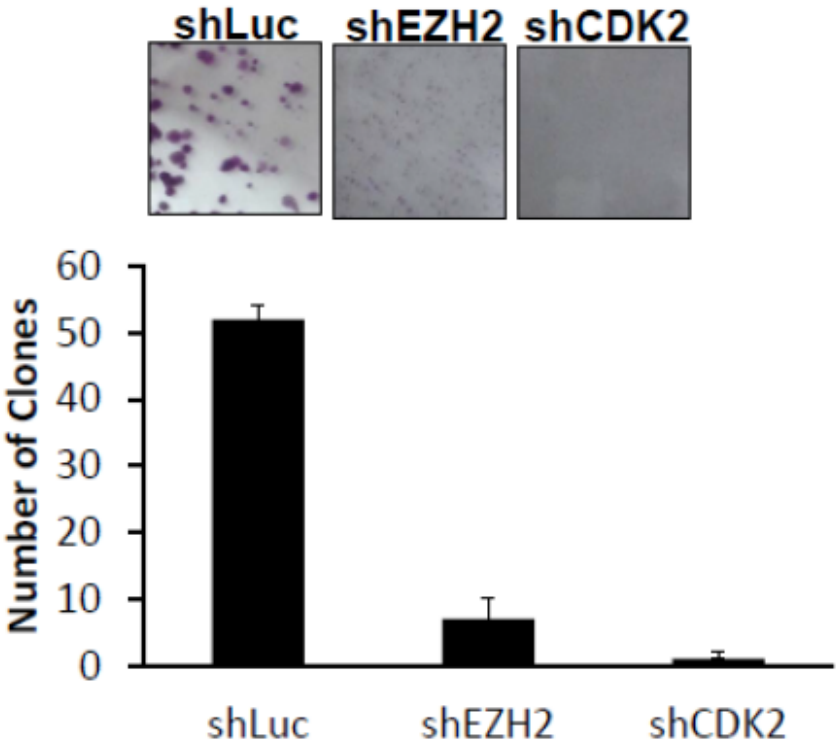
MDA-MB 231



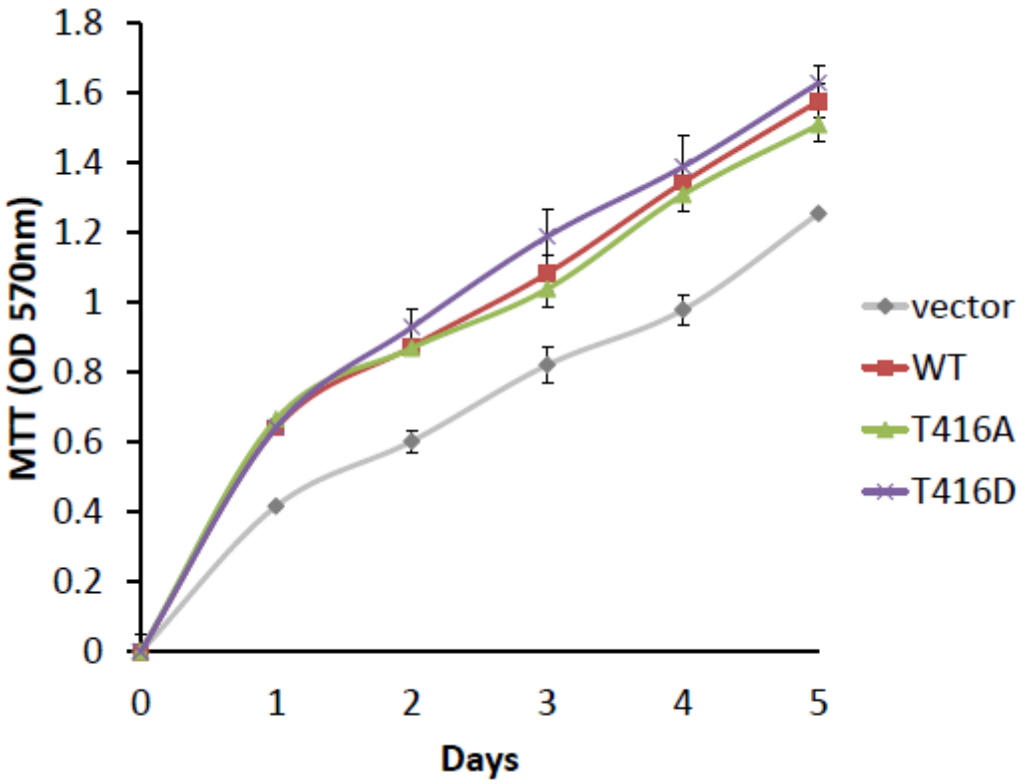
B


Vector
WT
T416A
T416D
Number of Clones
80
70
60
50
40
30
20
10
0
vector
WT
T416A
T416D

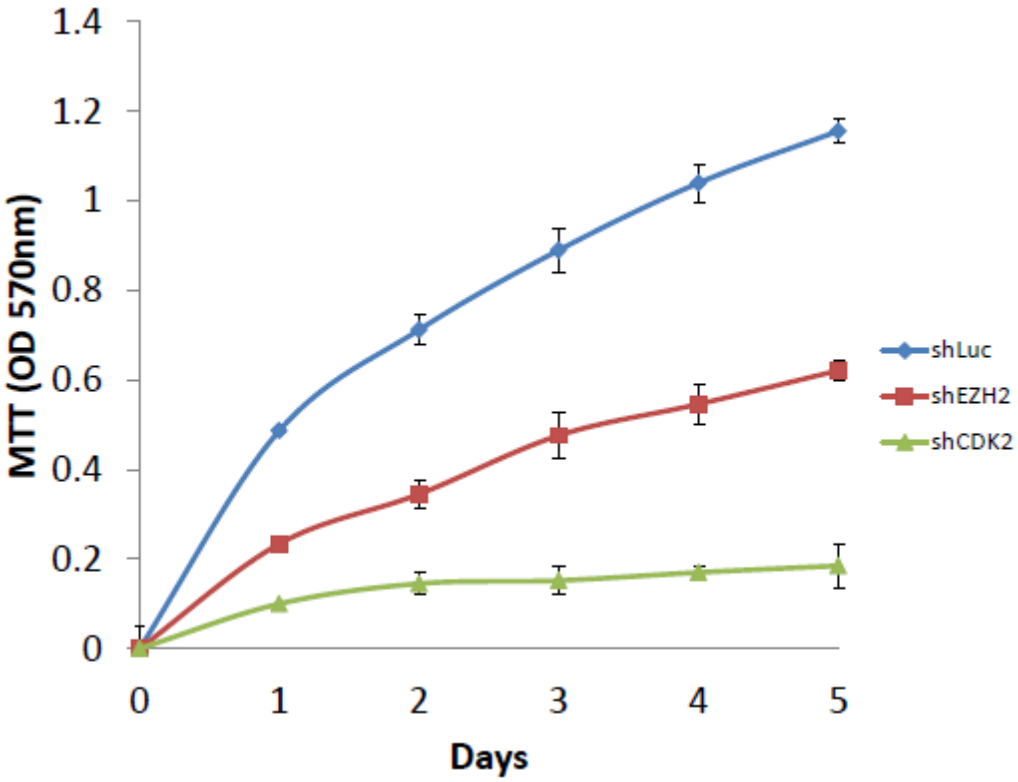
C


shLuc
shEZH2
shCDK2
Number of Clones
60
50
40
30
20
10
0
shLuc
shEZH2
shCDK2

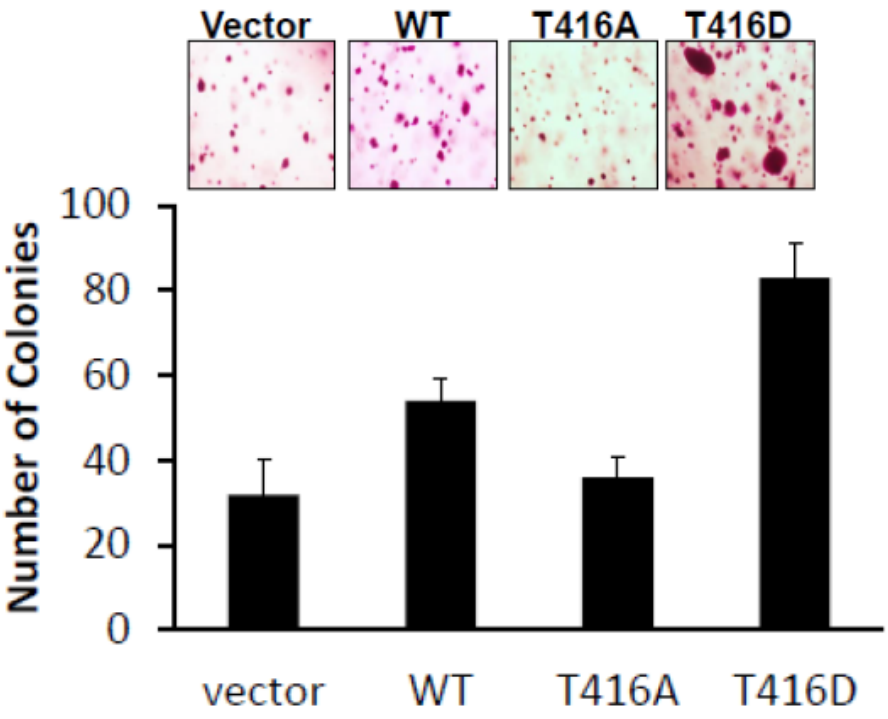
D


1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
MTT (OD 570nm)
0
1
2
3
4
5
Days
vector
WT
T416A
T416D

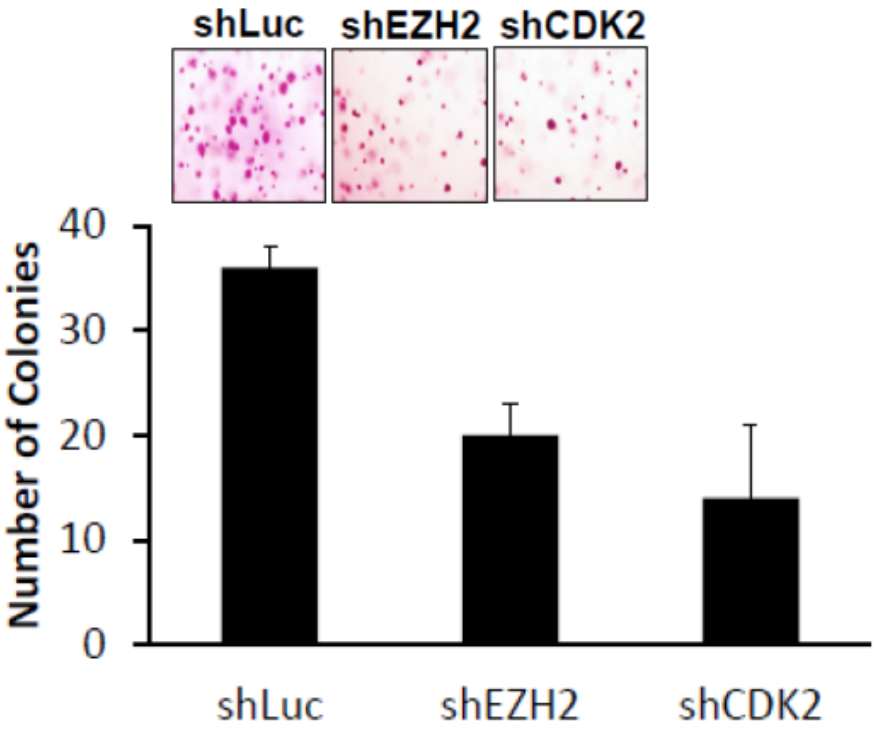
E


1.4
1.2
1
0.8
0.6
0.4
0.2
0
MTT (OD 570nm)
0
1
2
3
4
5
Days
shLuc
shEZH2
shCDK2

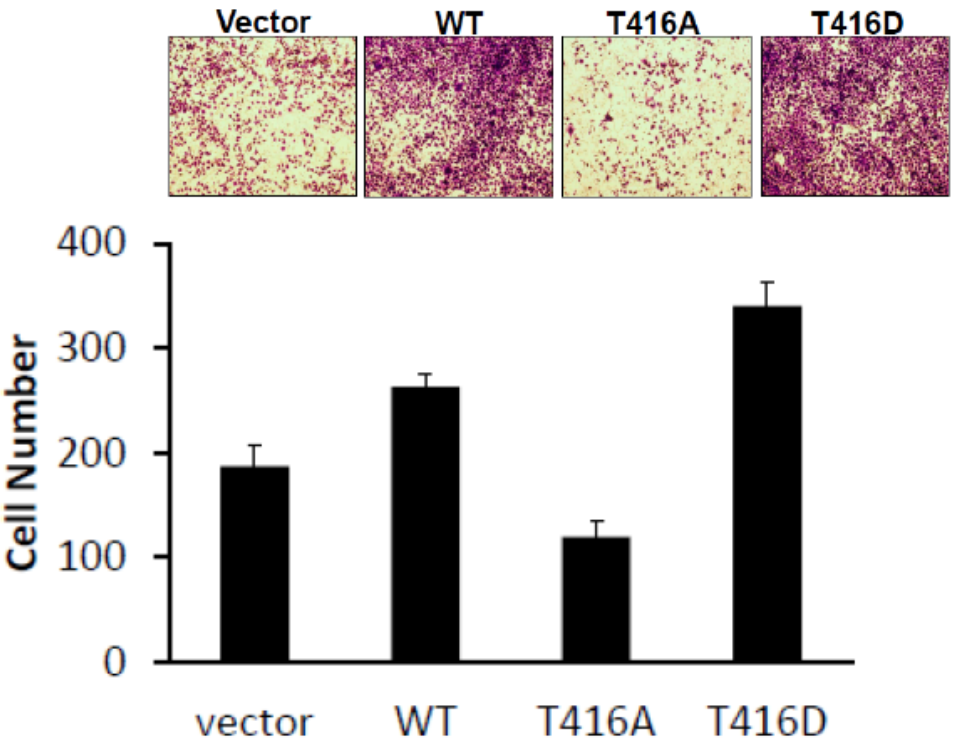
F


Vector
WT
T416A
T416D
Number of Colonies
100
80
60
40
20
0
vector
WT
T416A
T416D

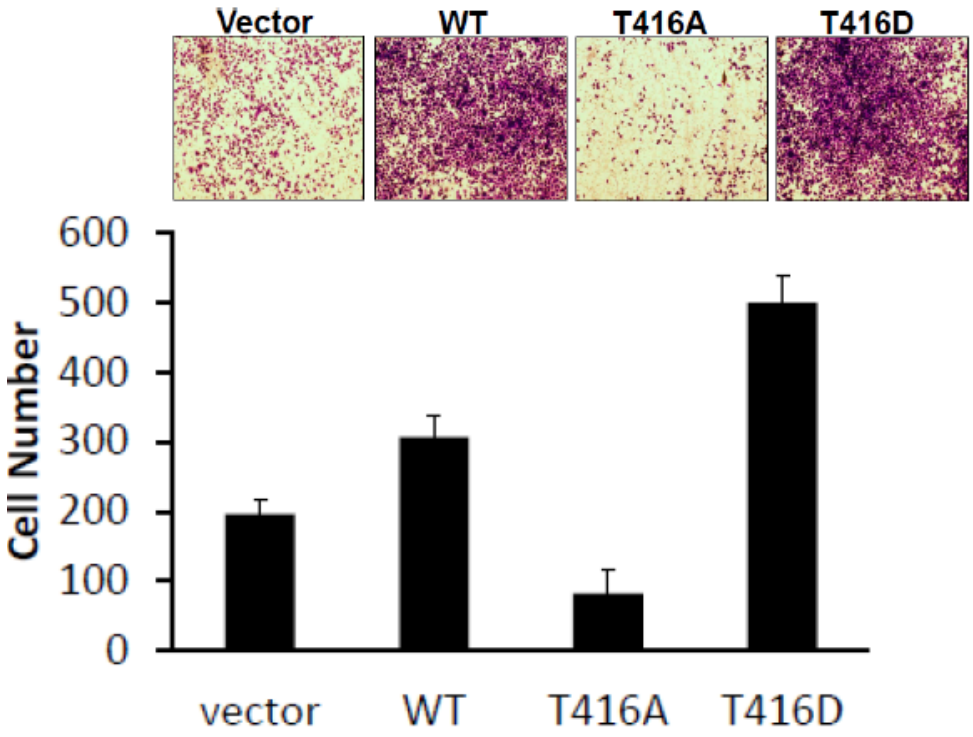
G


shLuc
shEZH2
shCDK2
Number of Colonies
40
30
20
10
0
shLuc
shEZH2
shCDK2

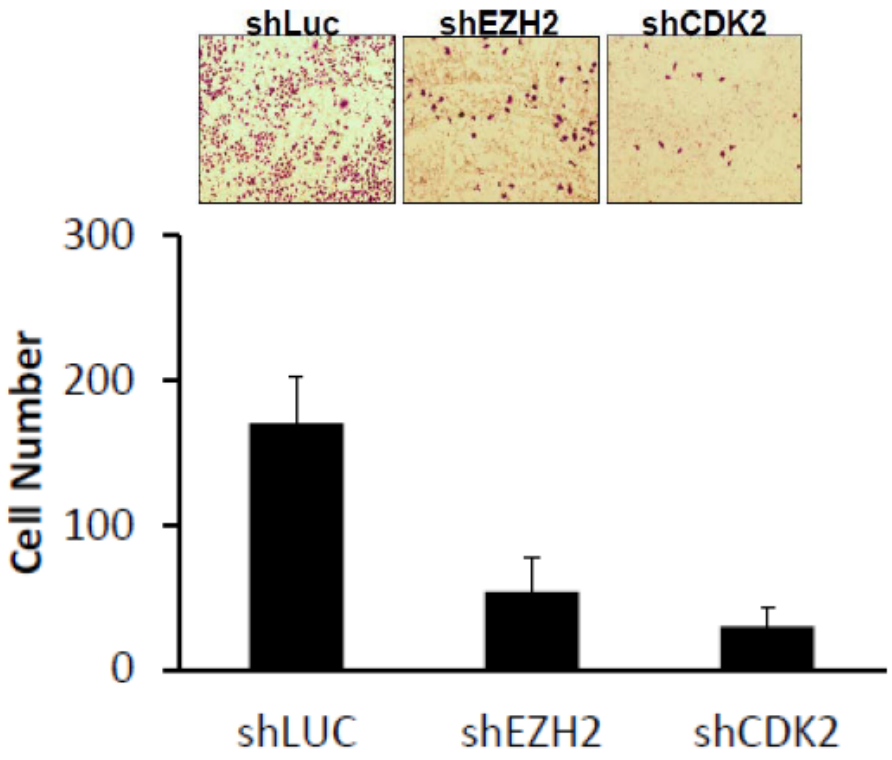
H


Vector
WT
T416A
T416D
Cell Number
400
300
200
100
0
vector
WT
T416A
T416D

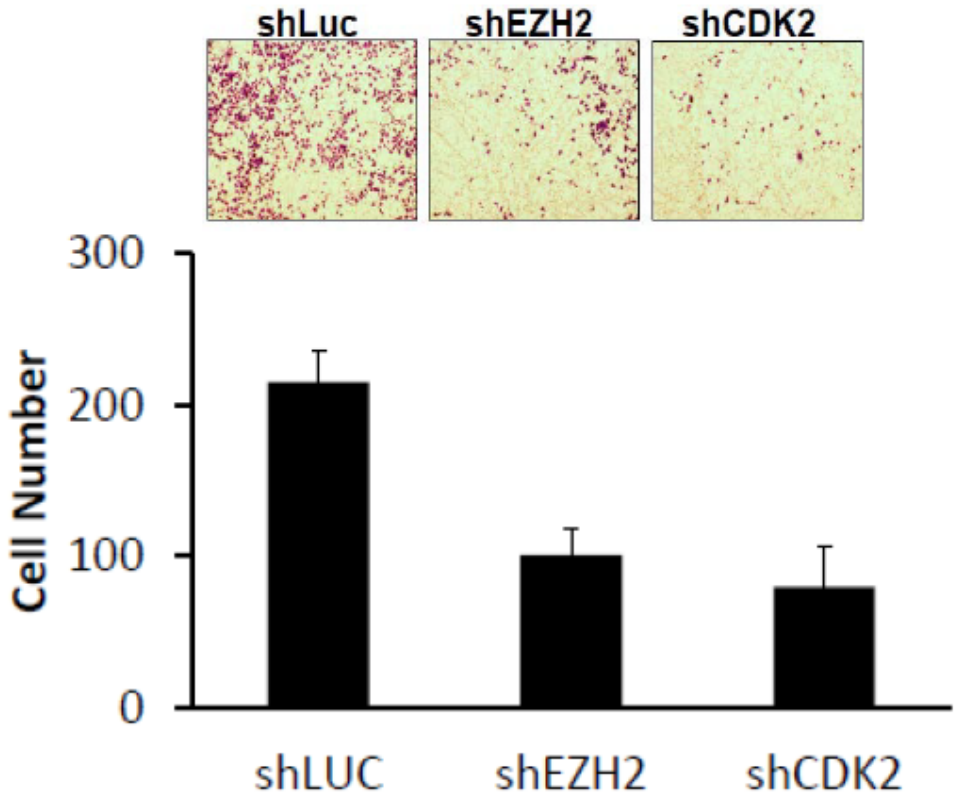
I


Vector
WT
T416A
T416D
Cell Number
600
500
400
300
200
100
0
vector
WT
T416A
T416D

J


shLuc
shEZH2
shCDK2
300
200
100
0
Cell Number
shLUC
shEZH2
shCDK2

K


shLuc
shEZH2
shCDK2
300
200
100
0
Cell Number
shLUC
shEZH2
shCDK2

4.6.4 EZH2 T416 phosphorylation enhances breast cancer tumor sphere formation

Many types of cancer stem and progenitor cells, including breast cancer cells have demonstrated EZH2 to be fundamental in the biology and in promoting the expansion of upper lineage cell populations. How EZH2 regulates each of these respective tumor initiating cells populations has been studied in many laboratory setting, but the signaling transduction mechanisms that regulate EZH2 in these cell populations or how these signal transduction events regulate EZH2 to generate or maintain these cell populations is yet to be elucidated. Knowing that EZH2 T416 phosphorylation was able to enhance colony formation in 3-D growth dependent manner based on T416D point mutation we asked if EZH2 T416 phospho mimetic (D-form) or phosphor-null (A-form) EZH2 variants can influence tumor sphere formation when the stable lines mentioned in 4.6.1 are seeded 10,000 cells/well in a non-adherent culture dish with the cells cultured in Mammocult media (Stem Cell Tech. Inc). After 7-10 days in the above culture media it was observed that tumor spheres formed by EZH2-WT expressing cells compared to those formed by A-form expressing cells had a 2-fold reduction in the number of tumor spheres formed. Moreover, the D-form expressing cells increased their number of spheres formed compared to EZH2-WT expressing cells by 2-fold, demonstrating EZH2 T416 phosphorylation can promote the formation of BLBC/TNBC tumor spheres. Sphere formation was confirmed to be both CDK2 and EZH2 dependent as shCDK2 cells had 9-fold reduction in tumor sphere number formation and shEZH2 cells had a 22-fold reduction in tumor

sphere formation, respectively. To determine if the elevated sphere formation was due to increased stem cell or progenitor cell population expansion, the respective cell populations were stained for changes in CD44/CD49f positive and CD24 /EPCAM negative cell populations. Cells were also stained using Aldefluor assay (Stem Cell Tech. Inc) to look at ALDH1 activity. After staining no apparent differences were seen in cell surface marker expression or ALDH1 activity between the stable lines expressing EZH2-WT, A-form, or D-form (data not shown), suggesting the increase in tumor sphere formation number is not due to an expansion in the breast cancer stem cell population. However this may be suggestive of the increase in sphere number being alternatively derived from a tumorigenic breast cancer progenitor cell population, but we were unable to identify the population by representative cell surface marker characterization.

4.6.5 EZH2 T416 phosphorylation enhances xenograft tumor growth

The observation that EZH2 T416 phosphoryaltion leading to increased 3-D growth an invasion potential asked the question whether or not EZH2 T416 phosphoryaltion has the ability promote *in vivo* tumor growth. The stable lines mentioned in 4.6.1, including EZH2 and CDK2 knockdown cell lines, were therefore evaluated in their ability to produce tumors in a xenograft mouse model. Each respective cell line was injected into a nude female mouse mammary fat pad using $2.0X10^6$ cells per injection. It was determined that EZH2 T416 phosphorylation increases *in vivo* tumorigenecity as the EZH2 A-form cell line tumor growth was mitigated and the EZH2 D-form tumor growth was enhanced

compared to EZH2-WT expressing cells. CDK2 and EZH2 knockdown cells both mitigated tumor growth. Taken together this demonstrates EZH2 T416 phosphoryaltion is candidate residue capable of enahncing tumor growth when T416 phosphorylation is mimicked by T416D point mutation and that T416 phosphorylation thus serves as a potential target for phamacological inhibition that can reduce BLBC tumor growth.

4.6.6 CDK2 inhibitor or EZH2 inhibitor reduces tumor sphere formation number

Both aberrant or hyperactivation of CDKs (i.e. CDK2) and EZH2 (through PRC2 function) has been shown to favor tumor development via expansion of cancer stem or progenitor cells through unscheduled cell division in either of these upper lineage breast cancer cell populations. Therefore inhibition of CDK2 or EZH2 may be a suitable intervention and drugable strategy for treating BLBC. Based on acquired knowledge that EZH2 T416 phosphorylation can enhance tumor sphere growth we developed a tumor sphere drug killing assay using BLBC-derived spheres as tissue culture test spheres. The rationale is to develop a target therapy to target progenitor cells or stem cells from the tumor. Tumor stem cell or progenitor cells are possessive of plasticity elements capable of generating or regenerating tumor biology heterogenic hierarchy components and are the cells resistant to conventional and known BLBC/TNBC proposed therapies. Therefore, if candidate therapy regimens show positive results in the sphere assay derived from BLBC/TNBC derived cell lines it may provide a

through-put design to test and optimize CDK or EZH2 based therapy treatment plans. Further validation can be achieved *in vivo* after initial sphere assay screening.

Figure 10. T416 phosphorylation enhances tumor sphere formation number and xenograft tumor growth and sphere number can be inhibited by SNS032 or GSK126

A. Tumor sphere assay using Mammocult media with V and EZH2 -WT, A, D-forms of MDA-MB 231 expression cells

B. Tumor sphere assay using Mammocult media with shLuc, shEZH2 and shCDK2 MDA-MB 231 cells.

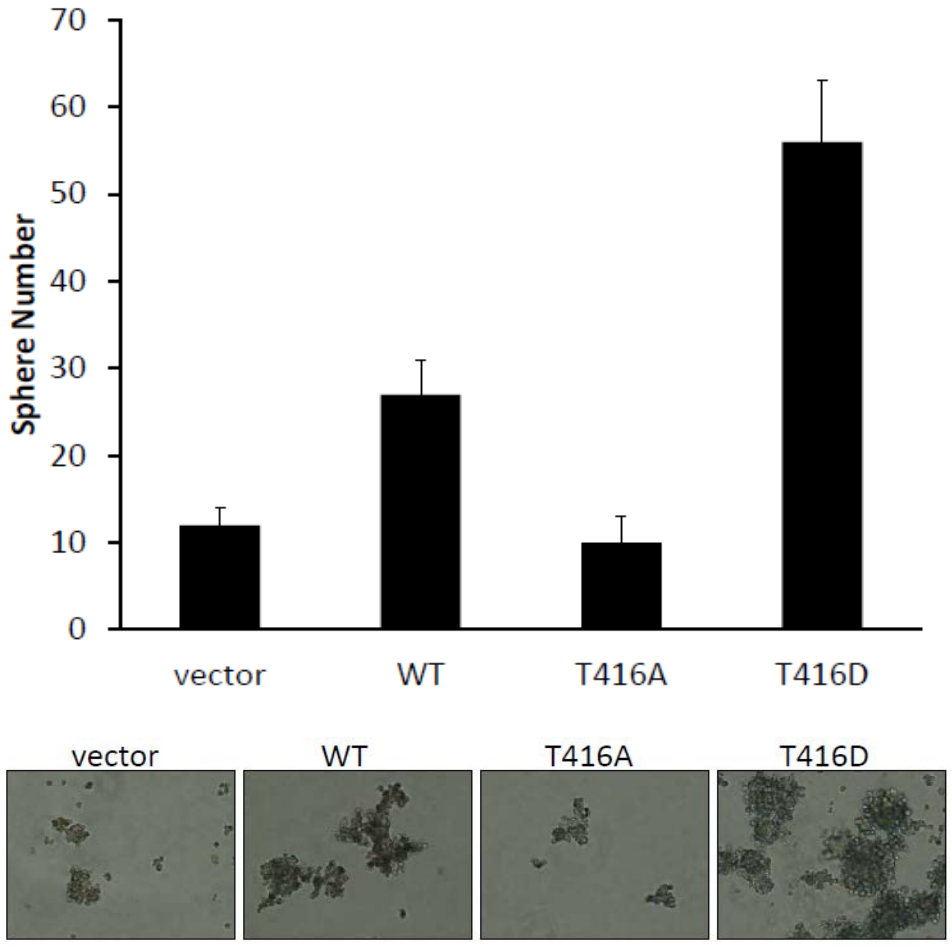
C. Xenograft tumor growth of with V and EZH2 -WT, A, D-forms of MDA-MB 231 expression cells

D. Xenograft tumor growth of with shLuc, shEZH2 and shCDK2 MDA-MB 231 cells.

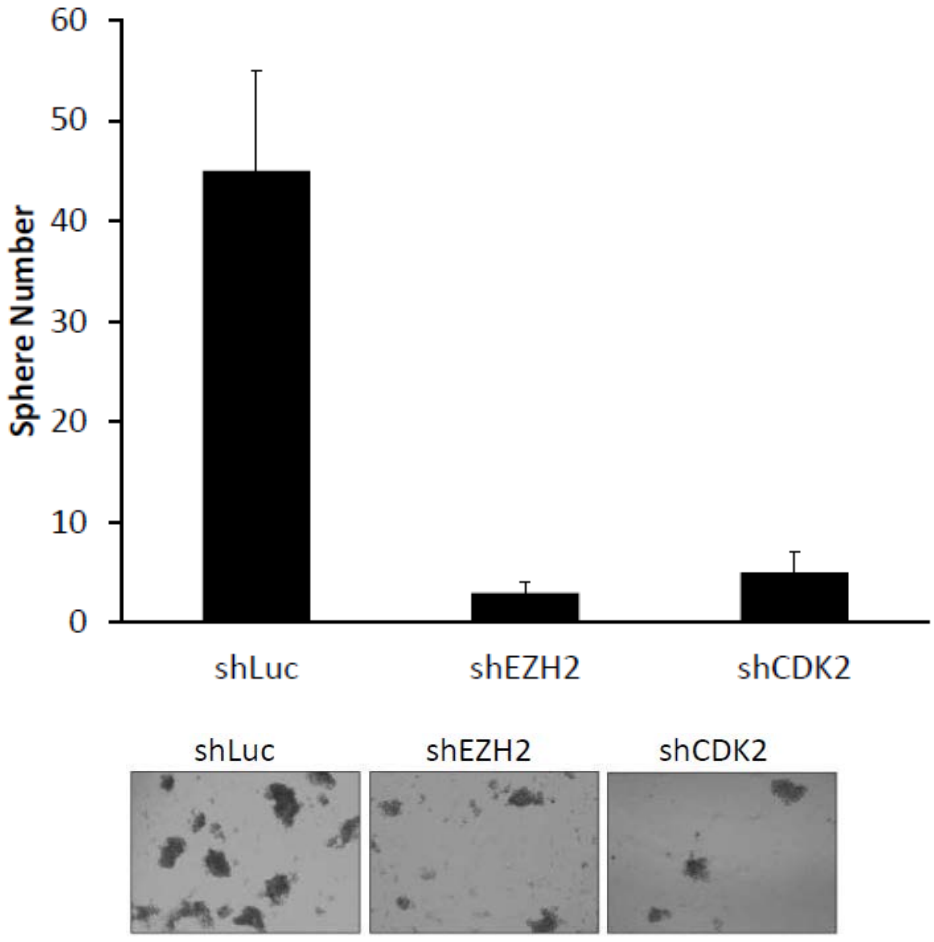
E**.** Tumor sphere inhibition by CDK2 inhibitor treatment with SNS032 or EZH2 inhibitor treatment with GSK126

Figure 10.

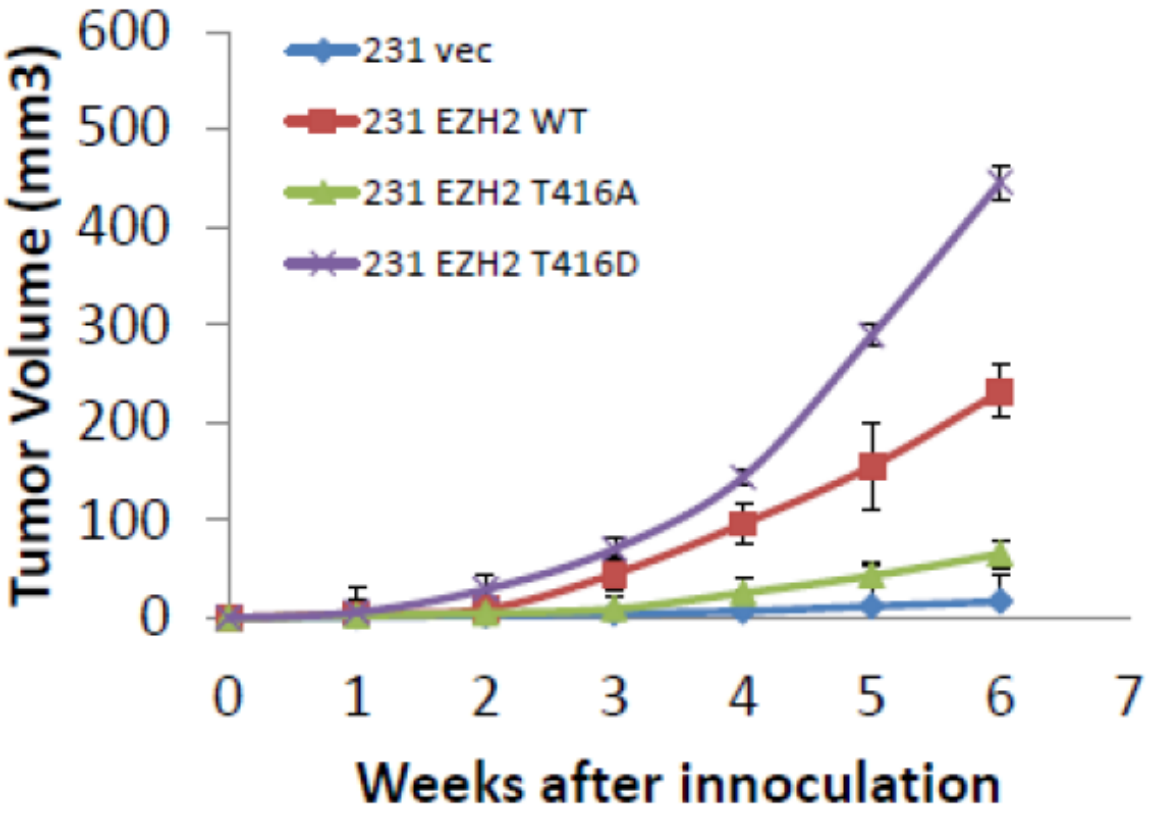
A


70
60
50
40
30
20
10
0
Sphere Number
vector
WT
T416A
T416D
vector
WT
T416A
T416D

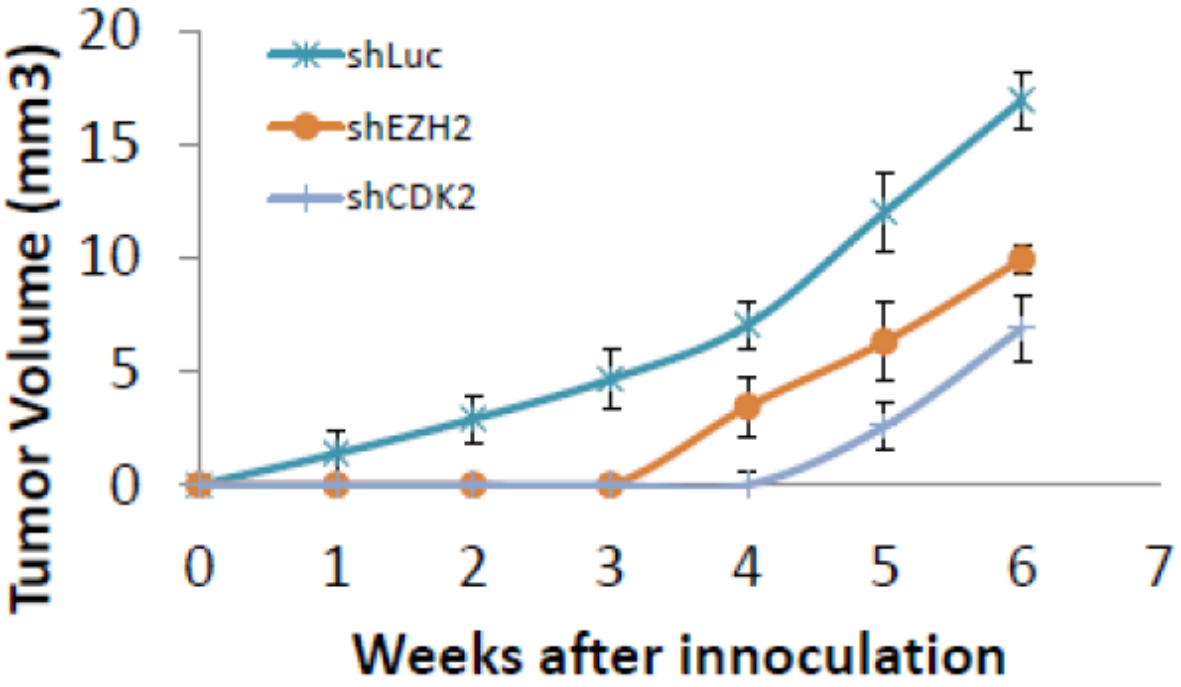
B


60
50
40
30
20
10
0
Sphere Number
shLuc
shEZH2
shCDK2
shLuc
shEZH2
shCDK2

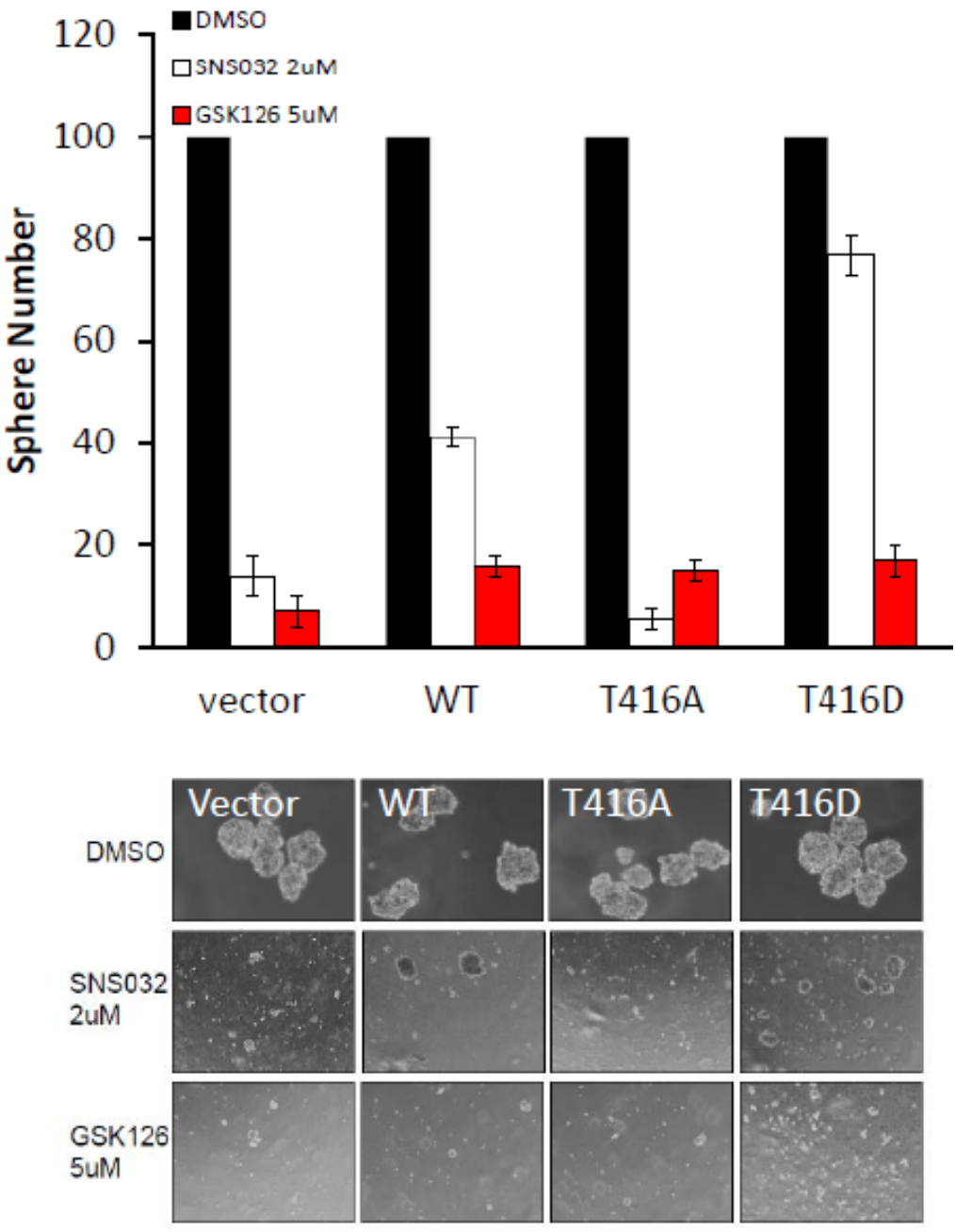
C


Tumor Volume (mm3)
600
500
400
300
200
100
0
231 vec
231 EZH2 WT
231 EZH2 T416A
231 EZH2 T416D
0
1
2
3
4
5
6
7
Weeks after innoculation

D


Tumor Volume (mm3)
20
15
10
5
0
shLuc
shEZH2
shCDK2
0
1
2
3
4
5
6
7
Weeks after innoculation

E


DMSO
SNS032 2uM
GSK126 5uM
Sphere Number
120
100
80
60
40
20
0
vector
WT
T416A
T416D
Vector
WT
T416A
T416D
DMSO
SNS032
2uM
GSK126
5uM

CHAPTER 5. DISCUSSION

The cellular architecture of the mammary tissue provides the mammary gland a dynamic system for tissue homeostasis. The structured composition makes the tumorigenic events occurring at the different layers in the hierarchical tissue environment of the breast develop tumors with a highly heterogenic nature. Mammary tumors may arise in different layers of the breast tissue, from the basal-layer epithelium enriched in populations of undifferentiated cell types or the luminal layers of more differentiated cell lineages, including terminally differentiated cell types. Tumors arising from the basal-layer are thought to be the origin of BLBC. These basal-layer derived tumors are hormone receptor-lineage unrestricted, meaning they do not express estrogen-alpha or progesterone receptors and are often similar to the gene expression profiled tumors of BLBC that were identified in the original subtype classification or via a PAM50 test. Often tumors of the basal origin are also negative for HER2 expression. In addition the tumors derived from this area of breast tissue are often enriched in upper lineage breast cancer stem or progenitor cells, a characteristic also observed in many BLBC/TNBCs. The expression of estrogen-alpha, progesterone, and HER2 receptors is the marker standard that provides a mammary oncologist with a classification scheme and a mode of treatment planning for the breast cancer subtype treatment based on the ER-alpha/PgR/HER2 expression. ER-alpha/PgR/HER2 markers are the most frequently targeted by current therapies and without their expression, such as with the case with BLBC, targeted therapies are very limited. Furthermore, BLBC

demonstrates high resistance levels to conventional chemotherapies making BLBC only treatable by putative care. Understanding the underlying principles that governs BLBC essential biology and therapy resistance will help in the development of new targeted therapies for BLBC/TNBC. Identification of new biomarkers and signaling pathways promise better care for BLBC/TNBC patients. New biomarkers may be regulated by essential signal transduction pathways previously identified in BLBC/TNBC. The biomarkers may give a read-out of the activity of such previously studied, but untreated pathways. In order to target these pathways we need to understand the level of crosstalk and how the pathway interactions promote the aggressive nature of BLBC/TNBC through regulation of the new biomarkers.

BRCA1 breast cancer is used as a BLBC/TNBC model because to date it is the most clinically relevant representation. Patients that have inherited BRCA1 mutations almost all develop tumors BLBC/TNBC characteristics, including a tumors with poorly differentiated cellular morphology and enrichment in cancer stem or progenitor cell populations. EZH2 overexpression has been shown to decrease the expression of BRCA1 protein and moreover cell lines derived from tumors resected from BRCA1 mutation carriers show a dependency on EZH2 expression. The EZH2 overexpression experiments show that ectopic addition of EZH2 also increases the size of the breast cancer stem cell niche. It is known that EZH2 overexpressing tumors and BRCA1 deficient tumors both developing undifferentiated breast cancers that both tumor types share similar gene signatures. The above mentioned evidence is suggestive of EZH2 playing a role

in modulating the biology of BLBC/TNBC in a manner either dependent or independent of EZH2 down-regulating BRCA1. Further study is needed to determine if EZH2 overexpression BLBC/TNBC tumors require BRCA1 loss to elicit their mode of tumorigenic action and phenotype development. It is known that EZH2 by one of two ways can cause the development of BLBC/TNBC or undifferentiated carcinomas (option 1) by maintaining cells in a non-differentiated state, including a stem cell state or (option 2) through a process of de-differentiation, most likely through EZH2-PRC2 dependent function; one route being through epigenetic repression or down-regulation of BRCA1. Both mechanisms are similar to the breast cancer stem or progenitor cell hypothesis, which as previously mentioned in 2011 Chang et al. demonstrated EZH2 can expand the breast tumor initiating cell or breast cancer stem cell populations. Whether the study by Chang et al was through a process of option 1 or option 2 is yet to be determined. None the less taken together this exhibits how EZH2 provides an important thread for the biology and architecture of BLBC/TNBC tumors, possibly through regulating breast cancer stem cell or progenitor cell populations.

Relatedly, cyclin E/CDK2 has been demonstrated to be involved in studies demonstrating involvement in the development and progression of BLBC/TNBC. For example high cyclin E expression is observed in BRCA1 mutation carriers and the high expression cyclin E negatively correlates with poor survival of the BRCA1 mutation carrier patients. Because CDK2 activity is directly reflected by cyclin E expression owing to the cyclin E being the activating adaptor protein of

CDK2, this demonstrates that the elevated cyclin E level is consequently increasing CDK2 activity therefore CDK2 kinase function likely plays a role in the development of these BLBC/TNBC tumors. This is further supported by not only high expression of cyclin E protein being observed in BRCA1-derived BLBCs, but also through the observation of the loss of the CDK2 inhibitor protein, p27, in these BRCA1-derived tumors resulting in elevated CDK2 activity. The elevation in CDK2 activity being important for BLBC/TNBC can most directly be observed in a constitutively active CDK2 fusion protein being capable of promoting the development of breast tumors with a basal-like component in an MMTV mouse model. Overall this data suggests that CDK2 activity and expression can promote the development of tumors with BLBC/TNBC phenotypic traits.

EZH2 and its PRC2 component SUZ12 have been shown to be transcriptionally regulated by Rb/E2F during the G1/S phase of the cell cycle. Both EZH2 and SUZ12 proteins exhibit peak expression during the G1/S transition checkpoint. At this same stage of the cell cycle CDK2 activity also peaks with continuous activity until the G2/M transition being elicited through activation by A-type cyclins. EZH2 has been shown to bind to and be phosphorylated by CDK1/CDK2 in a cell cycle dependent manner. This suggests that PRC2 activity and thus EZH2 activity may be enhanced through aberrant activation or deregulation of the cell cycle, specifically through CDK2 activity and CDK2 phosphorylation of EZH2 during the cell cycle. As mentioned aberrant PRC2 activity is synonymous with maintaining undifferentiated cell states with BLBC/TNBC gene signatures, an increased size in the niche pool of cancer stem

or progenitor cells and coincidently aggressive tumor traits representative of BLBC/TNBC. Understanding the underlying signaling events that generate the development and maintenance of the biology of BLBC through the deregulation of the cell cycle that leads to a hyperactivated PRC2, specifically the site of interest in this study, EZH2 T416 phosphorylation. Understanding EZH2 T416 phosphorylation and how it governs BLBC/TNBC biology will provide insight into the development of rationalized targeted therapies for mammary oncologist to make available for BLBC/TNBC patient care options.

5.1. Cell cycle regulatory role of the PRC2 through EZH2 T416 phosphorylation

It can be said the cell cycle stage where CDK2 activity is the highest at G1/S via E-type cyclins, is where EZH2 expression level peaks during the cell cycle. CDK2 activation by A-type cyclins sustains CDK2 activity throughout the cell cycle until G2/M, the point where EZH2 expression level decreases therefore from G1/S to G2/M EZH2 possesses the potential for being modulated by CDK2 T416 phosphorylation. Phosphorylation of EZH2 on T416 by CDK2 suggests a mechanism that could contribute to deregulated histone modification by the PRC2 gene silencing mechanisms in a cell cycle dependent manner in BLBC/TNBC. Elucidating how the epigenetic mosaic is altered in BLBC/TNBC may display further targets unveiled through CDK phosphorylation of EZH2. It is because cyclin-dependent kinases, CDK1 and CDK2 have been shown to control EZH2 function and both EZH2 and cyclin E /CDK2 expression negatively correlate with TNBC patient survival and progression, that an interesting question

was raised as to whether there is a functional relationship between cyclin E/CDK2 and EZH2 in the relation to the generation and maintenance of TNBC. Cohorts of 122 primary TN and 125 non-TN breast tumor tissues stained for cyclin E and EZH2 revealed that a high cyclin E expression level closely correlates with elevated EZH2 expression in the TNBC ($p<0.0001$). When the non-TNBC cohort, did not show correlation between cyclin E and EZH2 expression ($p=0.53$) it suggested a possible relationship between Cyclin E and EZH2 protein expression in important for the biology of BLBC/TNBC. Recently several publications have shown that CDK2, the only known enzymatic partner of cyclin E, can physically associate with EZH2. We confirmed this interaction in BLBC/TNBC breast cancer cell lines via co-immunoprecipitation. CDK2 interaction domain of EZH2 was mapped using *in vitro* translated S^{35} labeled HA-CDK2 co-incubated with GST-amino terminus fused EZH2 fragments representative of either EZH2 function domain I (amino acid residues 1-333), domain II (amino acid residues 334-610), or domain III, the SET domain (amino acid residues 611-746) and revealed that CDK2 predominantly associated with domain II of EZH2. An *in vitro* protein kinase assay performed via incubation of cyclinE-CDK2 complex with each of the GST-EZH2 fusion proteins showed domain II was the only domain phosphorylated by cyclin E/CDK2. Interestingly, domain II contains an evolutionary conserved CDK2 phosphorylation motif (K(R)S(T)PXK(R), where X is any residue. T416A mutagenesis mitigated CDK2 phosphorylation *in vitro* within domain II and in ectopically expressed full-length EZH2-T416A when compared to EZH2-WT in the presence of overexpressed

cyclin E suggesting T416 is likely to be the major site phosphorylated by CDK2. The *in vitro* protein kinase assay performed using GST-EZH2 domains was confirmed via incubation of cyclinE-CDK2 complex with GST-EZH2 full-length fusion proteins to demonstrate the mapping of the T416 phosphorylation site was not an artifact of protein truncation. The T416A mutation has been reported to reduce EZH2 phosphorylation *in vitro*, but the function of EZH2 T416 phosphorylation (pT416) has yet to be elucidated. To investigate whether pT416 exists *in vivo* we generated a mouse monoclonal antibody against the pT416 containing peptide which was characterized using a peptide dot blot assay to determine its specificity and sensitivity. Ectopic expression of cyclin E strongly enhanced the pT416, while phosphorylation of EZH2-T416A was virtually undetectable. Consistently, pT416 was not detectable in cells expressing DN-CDK2^{D146N} or cells treated with three different CDK2 specific inhibitors, indicating CDK2 is the kinase that phosphorylates T416 on EZH2.

To study the cell cycle dependency of the T416 EZH2 phosphorylation we intended to establish a cell culture system of synchronized cell populations and monitor pT416 as cells were released from their synchronization block and progressed through the cell cycle in a uniform manner. Hela cells, a canonical cell line model for cell cycle synchronization, were used in addition to the candidate TNBC cell line MDA-MB 231 cells. Cells were synchronized using double thymidine-block to achieve %80 cell cycle synchronization in G1/S phase. After synchronization cells were released from their cell cycle block and let to progress through the cell cycle in a synchronized manner. Both Hela cells and

MDA-MB 231 cells showed peak T416 phosphorylation of EZH2 during G1/S paralleling the peak expression of cyclin E. As expression of cyclin E decreased progressing toward the entry of G2/M, the pT416 also decreased. Notably EZH2 total protein level was also being reduced as the pT416 decreased. Half-life experiments with EZH2 and cyclin E ectopic co-expression in 293T cells determined T416 phosphorylation did not enhance EZH2 protein stability. Hela and MDA-MB 231 cells were also synchronized in either G1/S or G2/M using double-thymidine block and nocodazole block, respectively. Cells synchronized in G1/S demonstrated increased pT416 compared to unsynchronized cells. An increase in pT416 was not observed in cell synchronized with nocodazole. Taken together, these data indicate CDK2 binds to and phosphorylates EZH2 on T416 and pT416 is regulated in a cell cycle dependent manner and demonstrate the potential for T416 phosphorylation by EZH2 to control PRC2 gene silencing when compared to other precedent EZH2 phosphorylation events that demonstrated similar mechanisms after phosphorylation

5.2. Clinical significance and tumorigenic functions of EZH2 T416 phosphorylation

To investigate the clinical significance of EZH2 T416 phosphorylation the monoclonal antibody for EZH2 T416 phosphorylation was used to immunohistochemical stain cohorts of 122 primary TN and 125 non-TN breast tumor tissues. Validation of the phospho-T416 EZH2 (pT416-EZH2) antibody was performed using peptide competition assays. Examination of the TNBC cohort demonstrated that when T416 phosphorylation of EZH2 was elevated

there was a decreasing trend for patient survival with statistical significance ($p=0.049$). The non-TNBC cohort did not exhibit a distinguishable survival trend ($p>0.05$) Taken together this suggest T416 phosphorylation of EZH2 can serve as prognostic biomarker to predict TNBC survival. This phosphorylation serving as a biomarker can be directly linked with cyclin E expression and CDK2 activity by establishing the co-expression significance of either cyclin E or CDK2/p-CDK2 with EZH2 T416 phosphorylation. In this manner the clinical significance of T416 phosphorylation can be demonstrated to be potentially dependent upon CDK2 activity and yield rationale for the development of a CDK2 inhibitor based therapy for TNBC patients exhibiting the EZH2 T416 phosphorylation biomarker.

Moreover, this TNBC cohort staining asks whether EZH2 T416 phosphorylation can predict TNBC metastasis potential or predict other functions of TNBC related to known EZH2 oncogenic traits based on the modulation of T416 phosphorylation. Biomarkers to predict and diagnose TNBC behavior are not readily available. Further understanding of the TNBC biology that is driven by EZH2 T416 phosphorylation thus will allow for a better understanding of how tumors are developing under this post-translational modification of EZH2. The type of mammary tumors developed under T416 phosphorylation will allow oncologist to combine CDK2 inhibitor based regimens with other conventional and existing therapies based on the related behavior predicted by EZH2 T416 phosphorylation. To test whether pT416-EZH2 induced a BLBC with enhanced tumorigenesis, we examined TNBC functional hallmarks that overlap with known EZH2 function including (1) increased cell proliferation, (2) anchorage-

independent growth, and (3) migration/invasion in MDA-MB 231-vector, MDA-MB 231-EZH2-WT, MDA-MB 231-EZH2-T416A, and MDA-MB 231-EZH2-T416D stable clones generated via lentiviral infection. As reported EZH2 overexpression exhibited a change in cell proliferation based on colony formation assay results between the vector and EZH2 (WT, A, or D) cell lines but proliferation of MDA-MB 231 stable cell lines did not exhibit any change in proliferation capacity amongst the T416 phosphorylation wild-type or modulated cell lines MDA-MB 231-EZH2-WT, MDA-MB 231-EZH2-T416A, and MDA-MB 231-EZH2-T416D. Similar results were observed using MTT proliferation assay for the same cell lines. Reduced growth was observed MDA-MB 231-shCDK2 and MDA-MB 231-shEZH2 cells with knockdown of CDK2 or EZH2, respectively. Notably, a change in the growth of cells in anchorage independent fashion was observed when MDA-MB 231-EZH2-WT, MDA-MB 231-EZH2-T416A, and MDA-MB 231-EZH2-T416D cells were grown under soft-agar growth conditions or in 3D-growth conditions. Under these 3D-gorwth culture conditions MDA-MB 231-EZH2-T416A demonstrated reduced cell growth while the T416 phospho-mimetic cell line, MDA-MB 231-EZH2-T416D, demonstrated an increase in cell proliferation compared to MDA-MB 231-EZH2-WT. Similarly, MDA-MB 231-EZH2-T416A demonstrated reduced migration and invasion ability while the T416 phospho-mimetic cell line, MDA-MB 231-EZH2-T416D, demonstrated an increase in migration and invasion ability compared to MDA-MB 231-EZH2-WT. Tumor sphere formation assay demonstrated that in addition to the EZH2-T416D phospho-mimetic form of EZH2 inducing and the EZH2-T416A phospho-null

mimetic mitigating enhanced 3D-growth culture-dependent cell proliferation, migration, and invasion that T416 phosphorylation also promotes an increase breast cancer cell capacity to form increased tumor sphere numbers. MDA-MB 231-shCDK2 and MDA-MB 231-shEZH2 cells showed reduction in the aforementioned abilities of 3D-dependent culture growth, migration/invasion, and increased tumor sphere formation number demonstrating each function is dependent also upon specifically CDK2 or EZH2. Of interest there was not a notable increase in cell populations representative of breast cancer stem cells as seen by there existing no change in CD44, CD24, CD49F, EpCAM expressing populations or by cells displaying increased ALDH1 activity. This suggests that the increase in sphere formation was not due to enrichment of breast cancer stem cells, but instead indicative of a unidentified breast cancer progenitor cell population. Taken together this demonstrates that in endogenous cell lines EZH2 T416 phosphorylation can enhance breast cancer tumorigenecity in a manner indicative of traits observed to be exacerbated in BLBC. To establish *in vivo* relevance of EZH2 T416 phosphorylation orthotopic xenograft tumor models of MDA-MB 231-vector, MDA-MB 231-EZH2-WT, MDA-MB 23-EZH2-T416A, and MDA-MB 231-EZH2-T416D were generated via mammary fat pad injection. The orthotopic tumor models demonstrated reduced ability of MDA-MB 231-EZH2-T416A cells to induce tumor growth *in vivo* compared to MDA-MB 231-EZH2-WT. MDA-MB 231-EZH2-T416D cells were capable of increased tumor growth more MDA-MB 231-EZH2-WT (Figure 4E). MDA-MB 231-shLuc, MDA-MB 231-EZH2-shCDK2, and MDA-MB 231-EZH2-shEZH2 cells were also used as a control for

the orthotopic xenograft model. Knockdown of both CDK2 (MDA-MB 231-shCDK2) and EZH2 (MDA-MB 231-shEZH2) demonstrated reduction of *in vivo* tumor growth similar to MDA-MB 23-EZH2-T416A cells. As a result this data suggests that both in endogenous culture system and *in vivo* mouse models, that EZH2 T416 phosphorylation can present breast cancer cells with the ability to increase their tumorigenecity through a predicted PRC2 mode of action. Further ChIP-Sequencing studies need to be performed to specifically see how T416 phosphorylation alters breast cancer gene expression profiles.

5.3. Utilizing CDK2 inhibition to reduce tumor sphere growth in BLBC

During the past two decades an ongoing list of publications has exemplified the common occurrence in cancer cells of cell cycle deregulation. Many tumors acquire mutation hits that invoke constitutive mitogenic signaling combined or the inability to process anti-mitogenic signaling, both resulting in unchecked cell proliferation. In a contributing manner most tumors develop a level of genomic instability that can enhance the number and frequency of mutations as well as chromosomal instability, an alteration in the tumor cell's chromosomal number. Compounding of these cellular abnormalities creates a slippery slope increasing the susceptibility to the accrual of additional genetic shifts that lead to the development of tumor progression and more aggressive phenotypes through infinite genetic variation. These basic cell cycle defects of unchecked proliferation, genomic instability, and chromosomal instability are mediated both directly and indirectly by aberrant deregulation of CDKs. One of the underlying hallmarks of BLBC is genomic stability. The increased mutational

rate is one way that BLBC tumors are thought to acquire the hallmarks of their aggressive nature through the enhanced chance of genetic variation. Both EZH2 and CDK2 activity have been linked to the aid of BLBC/TNBC tumors acquiring genomic instability and consequent increased proneness to mutational genetic acquirements suggestive of their activity producing and being an essential component of breast cancer with the basal-like phenotype. Specifically aberrant or hyperactivation of CDKs (i.e. CDK2) or EZH2 through the PRC2 has been shown to favor tumor development via expansion of cancer stem or progenitor cells through unscheduled cell division in such upper lineage breast cancer cell populations. Therefore inhibition of CDK2 or EZH2 may be a suitable intervention and drugable strategy for treating BLBC. Based on acquired knowledge that EZH2 T416 phosphorylation can enhance tumor sphere growth we developed a tumor sphere drug killing assay using BLBC-derived spheres as cell line test subjects. The rationale being that progenitor cells or stem cells from the tumor are most resistant with plasticity elements capable of generating or regenerating tumor biology heterogenic hierarchy components that are resistant to conventional and known BLBC proposed therapies. Therefore, if candidate therapy regimens show positive results in the sphere assay it provides a through-put design to test and optimize CDK or EZH2 based therapy treatment plans. Further validation can be achieved *in vivo* after initial sphere assay screening. Drug developers have sought pharmacological inhibitors for various CDKs for decades. Despite attempts to synthesize inhibitors against specific CDKs most of the first and second round CDK drugs are pan-CDK inhibitors

exist owed to the high homology between CDK members. Some CDK drugs have progressed to Phase 2 and Phase 3 of clinical trials therefore CDK inhibitors show some promise. Matching the correct CDK inhibitor to the correct tumor type is important and also very challenging due to the pan-CDK inhibition and CDK-tumor dependency reported[108,110]. Alternative efforts have focused on generating antagonist peptides representative of specific CDK substrates. Short peptides representing native amino acid sequences representative of CDK2 phospho-sites competing with CDK from binding to and phosphorylating the endogenous protein. A 39 amino acid peptide named SPA310 representative of the retinoblastoma-like protein 2 (RBL2), a known CDK2 bona fide phosphorylation substrate, was designed utilizing this competitive antagonist strategy. Successfully SPA310 was able through its inhibitory effect on RBL2 phosphorylation by CDK2 inducing apoptosis and suppressing tumor growth[111]. Utilization of the native peptide used to generate our “in-house” custom T416 phospho-EZH2 antibody may provide a CDK2 substrate competitive peptide strategy similar to SPA310-RBL2 case. Synthesizing a linker sequence for cellular entry to increase membrane permeability would permit peptide entrance into the cell directed toward EZH2 canonical nuclear localization. Our study demonstrates EZH2 T416 phosphorylation has potential as a prognostic marker in BLBC and demonstrates the ability to promote tumorigenesis therefore by inhibiting EZH2 T416 phosphorylation through peptide competition we can provide a suitable treatment for BLBC patients exhibiting elevated levels of T416 phosphorylation. This presents an interesting hypothesis which requires further

development. Moreover our study does not rule out redundancy of other CDKs phosphorylating T416 on EZH2 therefore a pan-CDK inhibitor that can inhibit other CDKs capable of phosphorylating EZH2 in addition to CDK2 may still be a better therapeutic sword. Current CDK drugs in clinical trial and applied tumor type are listed in Table 5.

Table 5. Current clinical status of CDK inhibitors

Inhibitor	Targets	Clinical Trial	Sponsor
AG-024322	CDK1,CDK2 CDK4	Phase I, advanced cancer	Pfizer
AT-7519	CDK1,CDK2 CDK4,CDK5	Phase I/II, advanced or metastatic cancer	Astex
P276-00	CDK1,CDK4 CDK9	Phase I/II, refractory neoplasms	Piramal
P1446A-05	CDK4	Phase I/II, advanced refractory neoplasms	Piramal
PD0332991	CDK4,CDK6	Phase I, advanced cancer	Pfizer
R547	CDK1,CDK2 CDK4,CDK7	Phase I, advanced solid tumors	Holfmann-LaRoche
Roscovitine	CDK1,CDK2 CDK7,CDK8 CDK9	Phase II, non-small cell lung cancer, haematological cancer	Cyclacel
SNS032	CDK1,CDK2 CDK7,CDk9	Phase I/II B-lymphoid malignancies Phase I/II solid tumors	Sunseis

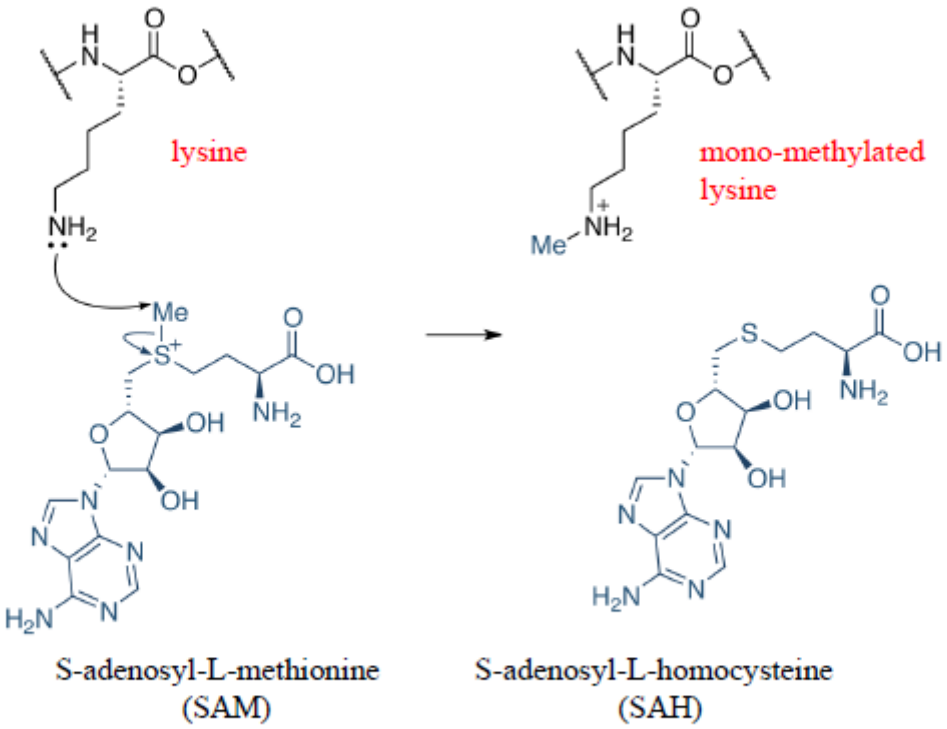
To assess the CDK2-dpendency on sphere growth MDA-MB 231-vector, MDA-MB 231-EZH2-WT, MDA-MB 23-EZH2-T416A, and MDA-MB 231-EZH2-T416D stable lines were cultured in Mammocult media from StemCell. Previously different capacity for sphere number was established based on modulation of the T416 EZH2 phosphorylation site therefore sphere number was normalized before adding chemical inhibitors for either CDK2 or EZH2. One of the most specific CDK2 inhibitors, SNS032 was used to treat spheres derived from MDA-MB 231 stable EZH2 cell lines. Roscovitine was used and showed similar results. Both inhibitors were effective at reducing sphere number after drug treatment for 7-10 days. Sphere number was reduced by %60 after SNS032 treatment compared DMSO control treatment. shCDK also reduced sphere number by a comparable margin compared to shLuc control. The MDA-MB 231-EZH2-T416D phospho-mimetic cell line for EZH2 T416 phosphorylation exhibited 3-fold more resistance to SNS032 treatment. This sphere killing assay data suggests BLBC-derived spheres number can be reduced by depleting cells of active CDK2. As spheres represent the most challenging cell population of breast cancer due to their intrinsic stem or progenitor cell characteristic this assay can serve a screen for the efficacy of other inhibitors in the treatment and reduction of breast tumor sphere numbers, such as treatment with other CDK2 inhibitors or EZH2 inhibitors.

5.4. Utilizing EZH2 inhibition to reduce tumor sphere growth in BLBC

EZH2 is the histone methyltransferase component of the polycomb repressive complex 2 (PRC2), which initiates transcriptional repression via histone 3 K27 tri-methylation (H3K27Me3) target gene promoter regions[8]. EZH2 expression correlates with advanced tumor stage and increased mortality specific to TNBC patients. EZH2 function has been shown to enhance tumor progression, metastasis, angiogenesis, and the population of breast tumor initiating cells. Therefore inhibiting tumor progression through specific inhibition of EZH2 has been a sought after therapeutic strategy for over a decade. The issue with developing a specific inhibitor against EZH2 is it's off target inhibition of other methyltransferases. Off target effects are wide-spread in the cell as protein lysine and arginine methyltransferases are ubiquitously expressed and serve to govern a vast array of cell functions. This off-target effect will reduce the efficacy of a therapy meant to treat cancers dependent on EZH2 as the decided directional target for the therapy. A cyclopentenyl derivative of 3-deazaadenosine called 3-deazaneplanocin A (DZNep) was the first line of EZH2 inhibitor following this trend, but as mentioned it has the such aforementioned broad scope of off target effects against other lysine and arginine methyltransferases. DZNep inhibits S-adenosyl-homocysteine (SAH) hydrolase, the enzyme needed for the conversion of SAH to adenosine and homocysteine via hydrolysis. This inhibition results in the intracellular accumulation of AdoHcy or SAH, which leads to inhibition of the S-adenosyl-l-methionine dependent lysine methyltransferase activity[82,112]. EZH2 is a member of the protein lysine methyltransferases (PKMTs) protein family that

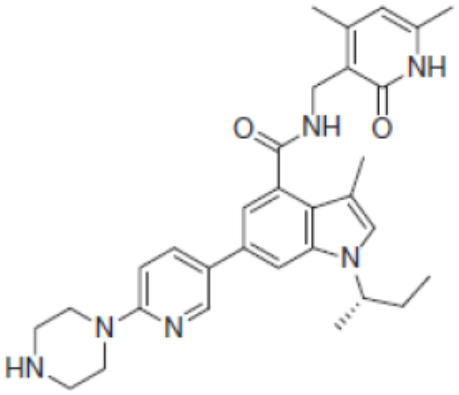
catalyzes methyl group transfer from s-adenosyl-l-methionine (AdoMet or SAM) to the epilson amine group of lysine. Figured 11depicts how SAM is converted to SAH resulting in lysine or arginine methylation. This process reduces the level of required PKMT substrate for lysine methylation and inhibits PKMTs. DZNep has been shown to reduce the levels of PRC2 component expression, including EZH2 and SUZ12, through what is thought to be transcriptional regulation in different types of cancer including breast cancer cells. As a result of the downregulation of the PRC2 components there is a concomitant loss of H3 lysine-27 trimethylation and a re-expression of PRC2 target genes. As a result, DZNep has shown capabilities to inhibit tumor development and induce tumor cell apoptosis in several cancer types[82,112,113]. At tumor-responsive doses, DZNep has been reported not to harm non-transformed cells[82], but this report is controversial as it was shown to be effective treating BRCA1-deficient basal-like breast cancer cells, but animal showed high levels of toxicity[114].

Figure 11. Protein methyl transferase (PMTs) enzymatic processing scheme of SAM conversion to SAH during substrate methylation.

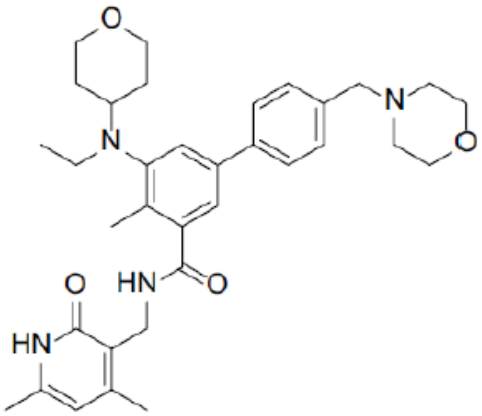


More specific EZH2 inhibitors would provide targeting EZH2 methyltransferase activity without reduction of PRC2 components with less cytotoxic effects. Both GlaxoSmithKline (GSK)[115] and Epizyme[116,117] have independently and successfully reported the development of such small molecule inhibitors of EZH2. These inhibitors have been test piloted by both drug companies in lymphomas with EZH2 activating mutations. GlaxoSmithKline named its inhibitor GSK126 or GSK343, and has made this available as part of the SGC epigenetics initiative. GSK343 inhibits EZH2 with an IC50 of 4nM and is 1000-fold more specific EZH2 than for other HMTs. It is 60-fold more selective for EZH2 in comparison to EZH1[115]. Epizyme reported results that were similar to GSK343 for its compound EPZ-6438. EPZ-6438 was 4500-fold more specific than for the other HMTs tested and 35-fold more selective against EZH1[116,117]. Both drugs notably reduce H3 lysine-27 trimethylation, but do not decrease expression of PRC2 components as seen with the first line of EZH2 inhibitor, DZNep. The chemical structure for GSK343 and EPZ-6438 can be seen in Figure 12. Moreover EPZ-6438 demonstrated good oral bioavailability in animals making it suitable for in vivo studies. No such report was available for GSK343 as of yet. Interestingly in June 2013 an EPZ-6438 based Phase 1 and Phase 2 clinical trial was initiated by Epizyme in patients with advanced solid tumors or with relapsed or refractory B-cell lymphoma. This is the second histone methyltransferases drug under clinical trial through Epizyme. The second drug under clinical development is called DOT1L. Epizyme granted Eisai a worldwide license to EPZ-6438 and in collaboration with Eisai and Roche to develop companion diagnostic tests.

Figure 12. Chemical structures of specific EZH2 inhibitors in the clinical trial pipeline



GSK126



EPZ-6438

In our study we wished to decipher how EZH2 inhibition using EZH2 specific inhibitors can reduce sphere number. Due to the timing of the study we used GSK126 because it was available for use through GSK. Further study should include using EPZ-6438 but at the beginning of the sphere killing assay EPZ-6438 was not available. To assess the EZH2-dpendency on sphere growth MDA-MB 231-vector, MDA-MB 231-EZH2-WT, MDA-MB 231-EZH2-T416A, and MDA-MB 231-EZH2-T416D stable lines were cultured in Mammocult media from StemCell. Previously different capacity for sphere number was established based on modulation of the T416 EZH2 phosphorylation site so sphere number was normalized before adding chemical inhibitors for EZH2. Both GSK126 and DZNep was used to treat spheres derived from MDA-MB 231-vector, MDA-MB 231-EZH2-WT, MDA-MB 231-EZH2-T416A, and MDA-MB 231-EZH2-T416D. DZNep data is not shown. Independently of T416 phosphorylation status the EZH2 inhibitor, GSK126 was able to reduce the sphere number by greater than %80. GSK126 treatment circumvented any resistance from the T416D cell line observed under SNS032 treatment. Taken together this suggests that EZH2 inhibitor, GSK126 is very effective at killing tumor spheres derived from the BLBC MDA-MB 231 candidate cell line. GSK126 should be tested in additional BLBC cell-derived sphere models.

5.5. Study summary and conclusion

In summary, basal like breast cancer has very limited targeted therapies available treatment. Other subtypes of breast cancer have available such targeted therapies that are based on molecular classification and therefore these types of presented tumors can be handled and is the reason why breast cancer mortality rates are on the decline. But because breast cancer is the most common type of tumor developed in women and %15-20 of breast cancer diagnosed is BLBC, which consequently makes these cases “untreatable”, a major endeavor for breast cancer researchers to undertake is in order to provide mammary oncologist with suitable treatment options is presented. Discovering new biomarkers for the prediction of the onset of BLBC tumor development, to provide new therapy targets tumor treatment, and to predict therapeutic resistance or response are all essential for learning how to treat BLBC patients. As mentioned this subtype of breast cancer is referred to as triple-negative breast cancer when the BLBC tumor lacks the expression of ERα, PgR and Her2. As reported and previously mentioned CDK activity and EZH2 epigenetic function is important for BLBC tumor biology. Moreover, phosphorylation of EZH2 by cyclin dependent kinases (CDK) has recently has been reported to control EZH2 epigenetic function consequently controlling cancer cell proliferation, invasion, and stem cell differentiation. By means of establishing that EZH2 and Cyclin E, the enzymatic activator of CDK2, co-expressed with clinical significance in triple-negative breast (TNBC) patient compared to normal breast cancer and that CDK2 phosphorylates EZH2 endogenously on residue T416 in breast cancer cell

lines in a cell cycle-dependent manner with observed clinical significance to reduction in TNBC patient survival exclusively in TNBC tissue we are able to propose EZH2-T416 phosphorylation (pT416) now as a potential therapeutic biomarker. Our study further concludes the functionality of pT416 as an enhancer of EZH2 to increase TNBC cell migration/invasion, mammosphere formation, and *in vivo* tumor growth. Mammosphere formation are both mitigated with administration of CDK2 clinical trial inhibitor SNS032 or GSK126 EZH2 specific inhibitor therefore, from our study we further postulate pT416 to be a therapeutic biomarker for aggressive forms of breast cancer and propose CDK2 or EZH2 inhibitor based therapies to reduce the size of the breast cancer stem or progenitor cell populations and tumor size. In conclusion, the components of this study describe a rationale to proceed to preclinical animal models with the perspective of later clinical studies for designing new therapeutic regimens of CDK based and EZH2 based inhibitors for the treatment of TNBC patients.

5.6. Future directions

The work from this study establishes T416 phosphorylation of EZH2 as prediction marker of poor survival in TNBC patients with endogenous cell line data supporting the role of T416 phosphorylation promoting an increase in breast cancer tumorigenesis. Development of a mammosphere killing test demonstrated to be a useful screening assay to establish a CDK2 based or EZH2 based inhibitor treatment can reduce mammosphere number in a cell culture setting. From this study there are three forward areas to pursue (1) development and validation of a preclinical mouse model for CDK2 and EZH2

BLBC therapies (2) identification of PRC2 gene targeting elicited by T416 phosphorylation (3) expansion of our kinase and EZH2 regulation protein knowledge during the cell cycle and in TICs. To validate the CDK2 and EZH2 therapies the drug treatments should be administered in xenograft mice harboring BLBC tumors. Monitoring of tumor growth can accomplished through labeling the tumor cells with fire-fly luciferase in order to increase the sensitivity of the assay. Tumor size in these models should decrease after SNS032 treatment concordantly with reduction in T416 phosphorylation observed in the tumor tissue or H3K27Me3 reduction observed after GSK126 treatment. Evaluation of BLBC tumor growth in such a xenograft model provides a more *in vivo* pre-clinical development strategy for the SNS032 or GSK126 drug regimen. Moreover to determine if T416 phosphorylation can promote the development of BLBC phenotypic tumors therefore in order to study what biology is regulated directly by EZH2 phosphorylation in an *in vivo* context we have generated a conventional transgenic mouse of the oncogenic activating form of EZH2 driven by MMTV-LTR promoter TgEZH2T416D, respectively. Early determination of tumor development demonstrated no tumors in these mice suggesting a second hit is necessary. Therefore this mouse strain will be crossed with BRCA1/P53 deficient mice or viral tumor induced mouse strains to study how T416 phosphorylation promotes tumor development *in vivo*. Mechanistically our study produced very little data determining the epigenetic profile that is regulated by T416 phosphorylation. In order to determine the epigenetic mosaic of tumor biology controlled by T416 phosphorylation we should employ the use of next

generation Chip-sequencing technologies. Global levels of histone transcriptional initiation, elongation, and repression (in this case H3K27Me3) will be determined using defined epigenetic histone modification Chip antibodies in cells with EZH2-WT, A-form, and D-forms stably expressed Changes in gene activation will be determined based on these histone modifications present in the target gene promoter region. The gene activation or inactivation will be organized into functional categorical subset groups. In this way between the three cellular contexts mentioned above the functional mosaics regulated by T416 phosphorylation can be determined and secondary targets for therapy can be developed and validated by shRNA knockdown of the identified targets. In addition to how EZH2 is modulated in its gene repression ability by T416 phosphorylation this study demonstrated that EZH2 is regulated during the cell cycle and there is potential for identifying other proteins that regulate EZH2 in a cell cycle dependent manner that can aid in the treatment of BLBC. BLBC has dependency on many CKDs therefore alternate molecules effect EZH2 can be used to gain BLBC fundamental insight for later prospecting potential therapies. Determining EZH2 interacting proteins that cell cycle dependent can be done synchronizing BLBC cells in respective cells stages and performing IP-Mass-Spec. Perhaps these changes in protein partners reflect a control mechanism that can keep the oncogenic activities of EZH2 in check through the cell cycle progression. This study also in trend with current literature depicts that post-translational modification (PTM) specifically by kinase phosphorylation can regulate EZH2 function and PRC2 targeting to respective gene loci. Our study

suggests that T416 phosphorylation can promote tumor sphere formation and it is known EZH2 overexpression can promote expansion of the breast cancer stem cell population. It is possible that PTM by phosphorylation can regulate EZH2 which then can modulate EZH2 oncogenic regulation of the breast cancer stem or progenitor cell population. Identification of kinase interacting partners for EZH2 in the for example the breast cancer stem population will elucidate kinase phosphorylation on EZH2 that is essential for regulating cancer stem cell biology and by targeting this phosphorylation by using kinase inhibitors we can then potentially reduce or eliminate the breast cancer stem cell niche. EZH2 kinase antibody microarray and IP-Mass spec has been utilized in our lab to identify these novel kinase partners of EZH2 in FACS sorted CD44-high/CD24-low cell populations. Comparison to the kinase interacting partners of EZH2 in non-breast cancer stem cells should further validate the biological importance of kinase partners that phosphorylate EZH2 in the breast cancer stem cell population preserving the oncogenic and therapy resistant nature of this upper lineage breast cancer cells. It is known that the sphere formation by these progenitor or stem cells from the tumors is a primary assay to decipher if cells are indeed upper lineage breast cancer cells. Therefore using the sphere killing assay can serve as a preliminary test to validate the importance of new identified EZH2 kinase binding partners capable of phosphorylating EZH2.

CHAPTER 6. REFERENCES

1 Coburn, N. G., Cady, B., Fulton, J. P., Law, C. & Chung, M. A. Improving size, lymph node metastatic rate, breast conservation, and mortality of invasive breast cancer in Rhode Island women, a well-screened population. *Breast cancer research and treatment* **135**, 831-837, doi:10.1007/s10549-012-2215-1 (2012).

2 Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E. & Forman, D. Global cancer statistics. *CA: a cancer journal for clinicians* **61**, 69-90, doi:10.3322/caac.20107 (2011).

3 Petrelli, F., Coinu, A., Cabiddu, M., Ghilardi, M., Lonati, V. & Barni, S. Five or more years of adjuvant endocrine therapy in breast cancer: a meta-analysis of published randomised trials. *Breast cancer research and treatment* **140**, 233-240, doi:10.1007/s10549-013-2629-4 (2013).

4 Higgins, M. J. & Baselga, J. Targeted therapies for breast cancer. *The Journal of clinical investigation* **121**, 3797-3803, doi:10.1172/JCI57152 (2011).

5 Lumachi, F. Targeted therapies in the treatment of breast cancer and localized sarcomas. *Current medicinal chemistry* **20**, 593-595 (2013).

6 Ferrari, P., Nicolini, A. & Carpi, A. Targeted therapies of metastatic breast cancer: Relationships with cancer stem cells. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* **67**, 543-555, doi:10.1016/j.biopha.2013.03.006 (2013).

7 Reisfeld, R. A. The tumor microenvironment: a target for combination therapy of breast cancer. *Critical reviews in oncogenesis* **18**, 115-133 (2013).

8 Mannello, F. Understanding breast cancer stem cell heterogeneity: time to move on to a new research paradigm. *BMC medicine* **11**, 169, doi:10.1186/1741-7015-11-169 (2013).

9 Mannello, F. & Ligi, D. Resolving breast cancer heterogeneity by searching reliable protein cancer biomarkers in the breast fluid secretome. *BMC cancer* **13**, 344, doi:10.1186/1471-2407-13-344 (2013).

10 Baird, R. D. & Caldas, C. Genetic heterogeneity in breast cancer: the road to personalized medicine? *BMC medicine* **11**, 151, doi:10.1186/1741-7015-11-151 (2013).

11 Montagna, E., Maisonneuve, P., Rotmensz, N., Cancello, G., Iorfida, M., Balduzzi, A., Galimberti, V., Veronesi, P., Luini, A., Pruneri, G., Bottiglieri, L., Mastropasqua, M. G., Goldhirsch, A., Viale, G. & Colleoni, M. Heterogeneity of triple-negative breast cancer: histologic subtyping to inform the outcome. *Clinical breast cancer* **13**, 31-39, doi:10.1016/j.clbc.2012.09.002 (2013).

12 Leong, A. S. & Zhuang, Z. The changing role of pathology in breast cancer diagnosis and treatment. *Pathobiology : journal of immunopathology, molecular and cellular biology* **78**, 99-114, doi:10.1159/000292644 (2011).

13 Engstrom, M. J., Opdahl, S., Hagen, A. I., Romundstad, P. R., Akslen, L. A., Haugen, O. A., Vatten, L. J. & Bofin, A. M. Molecular subtypes,

histopathological grade and survival in a historic cohort of breast cancer patients. *Breast cancer research and treatment*, doi:10.1007/s10549-013-2647-2 (2013).

14 Sotiriou, C., Neo, S. Y., McShane, L. M., Korn, E. L., Long, P. M., Jazaeri, A., Martiat, P., Fox, S. B., Harris, A. L. & Liu, E. T. Breast cancer classification and prognosis based on gene expression profiles from a population-based study. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 10393-10398, doi:10.1073/pnas.1732912100 (2003).

15 Wesolowski, R. & Ramaswamy, B. Gene expression profiling: changing face of breast cancer classification and management. *Gene expression* **15**, 105-115 (2011).

16 Colombo, P. E., Milanezi, F., Weigelt, B. & Reis-Filho, J. S. Microarrays in the 2010s: the contribution of microarray-based gene expression profiling to breast cancer classification, prognostication and prediction. *Breast cancer research : BCR* **13**, 212, doi:10.1186/bcr2890 (2011).

17 Abu Rabi, Z., Todorovic-Rakovic, N., Markicevic, M., Stamatovic, L., Vujasinovic, T. & Nikolic-Vukosavljevic, D. Change of influence of prognostic markers on metastasis free interval during and after adjuvant tamoxifen therapy in breast cancer patients. *Journal of B.U.ON. : official journal of the Balkan Union of Oncology* **18**, 321-327 (2013).

18 Nichols, M. The fight against tamoxifen resistance in breast cancer therapy: a new target in the battle? *Molecular interventions* **7**, 13-16, doi:10.1124/mi.7.1.4 (2007).

19 Castaneda, C. A. & Gomez, H. L. Targeted therapies: Combined lapatinib and paclitaxel in HER2-positive breast cancer. *Nature reviews. Clinical oncology* **6**, 308-309, doi:10.1038/nrclinonc.2009.68 (2009).

20 Griffin, D. A., Penprase, B. & Klamerus, J. F. Relative dose intensity--improving treatment and outcomes in early-stage breast cancer: a retrospective study. *Oncology nursing forum* **39**, E459-467, doi:10.1188/12.ONF.E459-E467 (2012).

21 Waddell, J. A. Treatment-experienced breast cancer: new pathways for improving survival. Introduction. *American journal of health-system pharmacy : AJHP : official journal of the American Society of Health-System Pharmacists* **65**, S2-3, doi:10.2146/ajhp080087 (2008).

22 Kao, K. J., Chang, K. M., Hsu, H. C. & Huang, A. T. Correlation of microarray-based breast cancer molecular subtypes and clinical outcomes: implications for treatment optimization. *BMC cancer* **11**, 143, doi:10.1186/1471-2407-11-143 (2011).

23 Teng, Y. H., Thike, A. A., Wong, N. S. & Tan, P. H. Therapeutic targets in triple negative breast cancer - where are we now? *Recent patents on anti-cancer drug discovery* **6**, 196-209 (2011).

24 Tate, C. R., Rhodes, L. V., Segar, H. C., Driver, J. L., Pounder, F. N., Burow, M. E. & Collins-Burow, B. M. Targeting triple-negative breast

cancer cells with the histone deacetylase inhibitor panobinostat. *Breast cancer research : BCR* **14**, R79, doi:10.1186/bcr3192 (2012).

25 Dreyer, G., Vandorpe, T., Smeets, A., Forceville, K., Brouwers, B., Neven, P., Janssens, H., Deraedt, K., Moerman, P., Van Calster, B., Christiaens, M. R., Paridaens, R. & Wildiers, H. Triple negative breast cancer: Clinical characteristics in the different histological subtypes. *Breast*, doi:10.1016/j.breast.2013.01.009 (2013).

26 Hedenfalk, I., Ringner, M., Ben-Dor, A., Yakhini, Z., Chen, Y., Chebil, G., Ach, R., Loman, N., Olsson, H., Meltzer, P., Borg, A. & Trent, J. Molecular classification of familial non-BRCA1/BRCA2 breast cancer. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 2532-2537, doi:10.1073/pnas.0533805100 (2003).

27 Zhou, L., Li, K., Luo, Y., Tian, L., Wang, M., Li, C. & Huang, Q. Novel prognostic markers for patients with triple-negative breast cancer. *Human pathology*, doi:10.1016/j.humpath.2013.03.021 (2013).

28 Lips, E. H., Mulder, L., Oonk, A., van der Kolk, L. E., Hogervorst, F. B., Imholz, A. L., Wesseling, J., Rodenhuis, S. & Nederlof, P. M. Triple-negative breast cancer: BRCAness and concordance of clinical features with BRCA1-mutation carriers. *British journal of cancer* **108**, 2172-2177, doi:10.1038/bjc.2013.144 (2013).

29 Mahamodhossen, Y. A., Liu, W. & Rong-Rong, Z. Triple-negative breast cancer: new perspectives for novel therapies. *Medical oncology* **30**, 653, doi:10.1007/s12032-013-0653-1 (2013).

30 Ebbert, M. T., Bastien, R. R., Boucher, K. M., Martin, M., Carrasco, E., Caballero, R., Stijleman, I. J., Bernard, P. S. & Facelli, J. C. Characterization of uncertainty in the classification of multivariate assays: application to PAM50 centroid-based genomic predictors for breast cancer treatment plans. *Journal of clinical bioinformatics* **1**, 37, doi:10.1186/2043-9113-1-37 (2011).

31 O'Toole, S. A., Beith, J. M., Millar, E. K., West, R., McLean, A., Cazet, A., Swarbrick, A. & Oakes, S. R. Therapeutic targets in triple negative breast cancer. *Journal of clinical pathology* **66**, 530-542, doi:10.1136/jclinpath-2012-201361 (2013).

32 De Laurentiis, M., Cianniello, D., Caputo, R., Stanzione, B., Arpino, G., Cinieri, S., Lorusso, V. & De Placido, S. Treatment of triple negative breast cancer (TNBC): current options and future perspectives. *Cancer treatment reviews* **36 Suppl 3**, S80-86, doi:10.1016/S0305-7372(10)70025-6 (2010).

33 Schneider, B. P., Winer, E. P., Foulkes, W. D., Garber, J., Perou, C. M., Richardson, A., Sledge, G. W. & Carey, L. A. Triple-negative breast cancer: risk factors to potential targets. *Clinical cancer research : an official journal of the American Association for Cancer Research* **14**, 8010-8018, doi:10.1158/1078-0432.CCR-08-1208 (2008).

34 Santarosa, M. & Maestro, R. BRACking news on triple-negative/basal-like breast cancers: how BRCA1 deficiency may result in the development of a

selective tumor subtype. *Cancer metastasis reviews* **31**, 131-142, doi:10.1007/s10555-011-9336-6 (2012).

35 Lim, E., Vaillant, F., Wu, D., Forrest, N. C., Pal, B., Hart, A. H., Asselin-Labat, M. L., Gyorki, D. E., Ward, T., Partanen, A., Feleppa, F., Huschtscha, L. I., Thorne, H. J., kConFab, Fox, S. B., Yan, M., French, J. D., Brown, M. A., Smyth, G. K., Visvader, J. E. & Lindeman, G. J. Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. *Nature medicine* **15**, 907-913, doi:10.1038/nm.2000 (2009).

36 Wright, M. H., Calcagno, A. M., Salcido, C. D., Carlson, M. D., Ambudkar, S. V. & Varticovski, L. Brca1 breast tumors contain distinct CD44+/CD24- and CD133+ cells with cancer stem cell characteristics. *Breast cancer research : BCR* **10**, R10, doi:10.1186/bcr1855 (2008).

37 Valentin, M. D., da Silva, S. D., Privat, M., Alaoui-Jamali, M. & Bignon, Y. J. Molecular insights on basal-like breast cancer. *Breast cancer research and treatment* **134**, 21-30, doi:10.1007/s10549-011-1934-z (2012).

38 Breiling, A., Sessa, L. & Orlando, V. Biology of polycomb and trithorax group proteins. *International review of cytology* **258**, 83-136, doi:10.1016/S0074-7696(07)58002-2 (2007).

39 Marx, J. Developmental biology. Combing over the Polycomb group proteins. *Science* **308**, 624-626, doi:10.1126/science.308.5722.624 (2005).

40 Levine, S. S., Weiss, A., Erdjument-Bromage, H., Shao, Z. H., Tempst, P. & Kingston, R. E. The core of the polycomb repressive complex is compositionally and functionally conserved in flies and humans. *Molecular and cellular biology* **22**, 6070-6078, doi:Doi 10.1128/Mcb.22.17.6070-6078.2002 (2002).

41 Kuzmichev, A., Margueron, R., Vaquero, A., Preissner, T. S., Scher, M., Kirmizis, A., Ouyang, X., Brockdorff, N., Abate-Shen, C., Farnham, P. & Reinberg, D. Composition and histone substrates of polycomb repressive group complexes change during cellular differentiation. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 1859-1864, doi:10.1073/pnas.0409875102 (2005).

42 Tavares, L., Dimitrova, E., Oxley, D., Webster, J., Poot, R., Demmers, J., Bezstarosti, K., Taylor, S., Ura, H., Koide, H., Wutz, A., Vidal, M., Elderkin, S. & Brockdorff, N. RYBP-PRC1 complexes mediate H2A ubiquitylation at polycomb target sites independently of PRC2 and H3K27me3. *Cell* **148**, 664-678, doi:10.1016/j.cell.2011.12.029 (2012).

43 Schoeftner, S., Sengupta, A. K., Kubicek, S., Mechtler, K., Spahn, L., Koseki, H., Jenuwein, T. & Wutz, A. Recruitment of PRC1 function at the initiation of X inactivation independent of PRC2 and silencing. *The EMBO journal* **25**, 3110-3122, doi:10.1038/sj.emboj.7601187 (2006).

44 Ku, M., Koche, R. P., Rheinbay, E., Mendenhall, E. M., Endoh, M., Mikkelsen, T. S., Presser, A., Nusbaum, C., Xie, X., Chi, A. S., Adli, M., Kasif, S., Ptaszek, L. M., Cowan, C. A., Lander, E. S., Koseki, H. &

Bernstein, B. E. Genomewide analysis of PRC1 and PRC2 occupancy identifies two classes of bivalent domains. *PLoS genetics* **4**, e1000242, doi:10.1371/journal.pgen.1000242 (2008).

45 Luis, N. M., Morey, L., Di Croce, L. & Benitah, S. A. Polycomb in stem cells: PRC1 branches out. *Cell stem cell* **11**, 16-21, doi:10.1016/j.stem.2012.06.005 (2012).

46 Margueron, R. & Reinberg, D. The Polycomb complex PRC2 and its mark in life. *Nature* **469**, 343-349, doi:10.1038/nature09784 (2011).

47 Hock, H. A complex Polycomb issue: the two faces of EZH2 in cancer. *Genes & development* **26**, 751-755, doi:10.1101/gad.191163.112 (2012).

48 Sarma, K., Margueron, R., Ivanov, A., Pirrotta, V. & Reinberg, D. Ezh2 requires PHF1 to efficiently catalyze H3 lysine 27 trimethylation in vivo. *Molecular and cellular biology* **28**, 2718-2731, doi:10.1128/MCB.02017-07 (2008).

49 Margueron, R., Li, G., Sarma, K., Blais, A., Zavadil, J., Woodcock, C. L., Dynlacht, B. D. & Reinberg, D. Ezh1 and Ezh2 maintain repressive chromatin through different mechanisms. *Molecular cell* **32**, 503-518, doi:10.1016/j.molcel.2008.11.004 (2008).

50 Shen, X., Liu, Y., Hsu, Y. J., Fujiwara, Y., Kim, J., Mao, X., Yuan, G. C. & Orkin, S. H. EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. *Molecular cell* **32**, 491-502, doi:10.1016/j.molcel.2008.10.016 (2008).

51 Kuzmichev, A., Jenuwein, T., Tempst, P. & Reinberg, D. Different EZH2-containing complexes target methylation of histone H1 or nucleosomal histone H3. *Molecular cell* **14**, 183-193 (2004).

52 Simon, J. A. & Kingston, R. E. Mechanisms of polycomb gene silencing: knowns and unknowns. *Nature reviews. Molecular cell biology* **10**, 697-708, doi:10.1038/nrm2763 (2009).

53 O'Meara, M. M. & Simon, J. A. Inner workings and regulatory inputs that control Polycomb repressive complex 2. *Chromosoma* **121**, 221-234, doi:10.1007/s00412-012-0361-1 (2012).

54 Zhang, Z., Jones, A., Sun, C. W., Li, C., Chang, C. W., Joo, H. Y., Dai, Q., Mysliwiec, M. R., Wu, L. C., Guo, Y., Yang, W., Liu, K., Pawlik, K. M., Erdjument-Bromage, H., Tempst, P., Lee, Y., Min, J., Townes, T. M. & Wang, H. PRC2 complexes with JARID2, MTF2, and esPRC2p48 in ES cells to modulate ES cell pluripotency and somatic cell reprogramming. *Stem cells* **29**, 229-240, doi:10.1002/stem.578 (2011).

55 Herz, H. M., Mohan, M., Garrett, A. S., Miller, C., Casto, D., Zhang, Y., Seidel, C., Haug, J. S., Florens, L., Washburn, M. P., Yamaguchi, M., Shiekhattar, R. & Shilatifard, A. Polycomb repressive complex 2-dependent and -independent functions of Jarid2 in transcriptional regulation in Drosophila. *Molecular and cellular biology* **32**, 1683-1693, doi:10.1128/MCB.06503-11 (2012).

56 Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R. S. & Zhang, Y. Role of histone H3 lysine 27 methylation in

Polycomb-group silencing. *Science* **298**, 1039-1043, doi:10.1126/science.1076997 (2002).

57 Schuettengruber, B. & Cavalli, G. Polycomb domain formation depends on short and long distance regulatory cues. *PloS one* **8**, e56531, doi:10.1371/journal.pone.0056531 (2013).

58 Wilkinson, F. H., Park, K. & Atchison, M. L. Polycomb recruitment to DNA in vivo by the YY1 REPO domain. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 19296-19301, doi:10.1073/pnas.0603564103 (2006).

59 Hagarman, J. A., Motley, M. P., Kristjansdottir, K. & Soloway, P. D. Coordinate regulation of DNA methylation and H3K27me3 in mouse embryonic stem cells. *PloS one* **8**, e53880, doi:10.1371/journal.pone.0053880 (2013).

60 Mendenhall, E. M., Koche, R. P., Truong, T., Zhou, V. W., Issac, B., Chi, A. S., Ku, M. & Bernstein, B. E. GC-rich sequence elements recruit PRC2 in mammalian ES cells. *PLoS genetics* **6**, e1001244, doi:10.1371/journal.pgen.1001244 (2010).

61 Siddique, H. R. & Saleem, M. Role of BMI1, a stem cell factor, in cancer recurrence and chemoresistance: preclinical and clinical evidences. *Stem cells* **30**, 372-378, doi:10.1002/stem.1035 (2012).

62 Crea, F., Paolicchi, E., Marquez, V. E. & Danesi, R. Polycomb genes and cancer: time for clinical application? *Critical reviews in*

oncology/hematology **83**, 184-193, doi:10.1016/j.critrevonc.2011.10.007 (2012).

63 Kridel, R., Sehn, L. H. & Gascoyne, R. D. Pathogenesis of follicular lymphoma. *The Journal of clinical investigation* **122**, 3424-3431, doi:10.1172/JCI63186 (2012).

64 Yoo, K. H. & Hennighausen, L. EZH2 methyltransferase and H3K27 methylation in breast cancer. *International journal of biological sciences* **8**, 59-65 (2012).

65 Chang, C. J. & Hung, M. C. The role of EZH2 in tumour progression. *British journal of cancer* **106**, 243-247, doi:10.1038/bjc.2011.551 (2012).

66 Pasini, D., Bracken, A. P., Jensen, M. R., Lazzerini Denchi, E. & Helin, K. Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. *The EMBO journal* **23**, 4061-4071, doi:10.1038/sj.emboj.7600402 (2004).

67 Cao, R. & Zhang, Y. SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. *Molecular cell* **15**, 57-67, doi:10.1016/j.molcel.2004.06.020 (2004).

68 Yu, H., Simons, D. L., Segall, I., Carcamo-Cavazos, V., Schwartz, E. J., Yan, N., Zuckerman, N. S., Dirbas, F. M., Johnson, D. L., Holmes, S. P. & Lee, P. P. PRC2/EED-EZH2 complex is up-regulated in breast cancer lymph node metastasis compared to primary tumor and correlates with

tumor proliferation in situ. *PloS one* **7**, e51239, doi:10.1371/journal.pone.0051239 (2012).

69 Bachmann, I. M., Halvorsen, O. J., Collett, K., Stefansson, I. M., Straume, O., Haukaas, S. A., Salvesen, H. B., Otte, A. P. & Akslen, L. A. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **24**, 268-273, doi:10.1200/JCO.2005.01.5180 (2006).

70 Chase, A. & Cross, N. C. Aberrations of EZH2 in cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **17**, 2613-2618, doi:10.1158/1078-0432.CCR-10-2156 (2011).

71 Raaphorst, F. M., Meijer, C. J., Fieret, E., Blokzijl, T., Mommers, E., Buerger, H., Packeisen, J., Sewalt, R. A., Otte, A. P. & van Diest, P. J. Poorly differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene. *Neoplasia* **5**, 481-488 (2003).

72 Knudsen, E. S., Dervishaj, O., Kleer, C. G., Pajak, T., Schwartz, G. F. & Witkiewicz, A. K. EZH2 and ALDH1 expression in ductal carcinoma in situ: Complex association with recurrence and progression to invasive breast cancer. *Cell cycle* **12**, 2042-2050, doi:10.4161/cc.25065 (2013).

73 De Brot, M., Rocha, R. M., Soares, F. A. & Gobbi, H. Prognostic impact of the cancer stem cell related markers ALDH1 and EZH2 in triple negative

and basal-like breast cancers. *Pathology* **44**, 303-312, doi:10.1097/PAT.0b013e3283534bcb (2012).

74 Arnes, J. B., Collett, K. & Akslen, L. A. Independent prognostic value of the basal-like phenotype of breast cancer and associations with EGFR and candidate stem cell marker BMI-1. *Histopathology* **52**, 370-380, doi:10.1111/j.1365-2559.2007.02957.x (2008).

75 Kim, J., Villadsen, R., Sorlie, T., Fogh, L., Gronlund, S. Z., Fridriksdottir, A. J., Kuhn, I., Rank, F., Wielenga, V. T., Solvang, H., Edwards, P. A., Borresen-Dale, A. L., Ronnov-Jessen, L., Bissell, M. J. & Petersen, O. W. Tumor initiating but differentiated luminal-like breast cancer cells are highly invasive in the absence of basal-like activity. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 6124-6129, doi:10.1073/pnas.1203203109 (2012).

76 Gonzalez, M. E., Li, X., Toy, K., DuPrie, M., Ventura, A. C., Banerjee, M., Ljungman, M., Merajver, S. D. & Kleer, C. G. Downregulation of EZH2 decreases growth of estrogen receptor-negative invasive breast carcinoma and requires BRCA1. *Oncogene* **28**, 843-853, doi:10.1038/onc.2008.433 (2009).

77 Agherbi, H., Gaussmann-Wenger, A., Verthuy, C., Chasson, L., Serrano, M. & Djabali, M. Polycomb mediated epigenetic silencing and replication timing at the INK4a/ARF locus during senescence. *PloS one* **4**, e5622, doi:10.1371/journal.pone.0005622 (2009).

78 Chang, C. J., Yang, J. Y., Xia, W., Chen, C. T., Xie, X., Chao, C. H., Woodward, W. A., Hsu, J. M., Hortobagyi, G. N. & Hung, M. C. EZH2 promotes expansion of breast tumor initiating cells through activation of RAF1-beta-catenin signaling. *Cancer cell* **19**, 86-100, doi:10.1016/j.ccr.2010.10.035 (2011).

79 Gonzalez, M. E., DuPrie, M. L., Krueger, H., Merajver, S. D., Ventura, A. C., Toy, K. A. & Kleer, C. G. Histone methyltransferase EZH2 induces Akt-dependent genomic instability and BRCA1 inhibition in breast cancer. *Cancer research* **71**, 2360-2370, doi:10.1158/0008-5472.CAN-10-1933 (2011).

80 Cao, Q., Yu, J., Dhanasekaran, S. M., Kim, J. H., Mani, R. S., Tomlins, S. A., Mehra, R., Laxman, B., Cao, X., Yu, J., Kleer, C. G., Varambally, S. & Chinnaiyan, A. M. Repression of E-cadherin by the polycomb group protein EZH2 in cancer. *Oncogene* **27**, 7274-7284, doi:10.1038/onc.2008.333 (2008).

81 Lu, C., Han, H. D., Mangala, L. S., Ali-Fehmi, R., Newton, C. S., Ozbun, L., Armaiz-Pena, G. N., Hu, W., Stone, R. L., Munkarah, A., Ravoori, M. K., Shahzad, M. M., Lee, J. W., Mora, E., Langley, R. R., Carroll, A. R., Matsuo, K., Spannuth, W. A., Schmandt, R., Jennings, N. B., Goodman, B. W., Jaffe, R. B., Nick, A. M., Kim, H. S., Guven, E. O., Chen, Y. H., Li, L. Y., Hsu, M. C., Coleman, R. L., Calin, G. A., Denkbas, E. B., Lim, J. Y., Lee, J. S., Kundra, V., Birrer, M. J., Hung, M. C., Lopez-Berestein, G. &

Sood, A. K. Regulation of tumor angiogenesis by EZH2. *Cancer cell* **18**, 185-197, doi:10.1016/j.ccr.2010.06.016 (2010).

82 Crea, F., Fornaro, L., Bocci, G., Sun, L., Farrar, W. L., Falcone, A. & Danesi, R. EZH2 inhibition: targeting the crossroad of tumor invasion and angiogenesis. *Cancer metastasis reviews* **31**, 753-761, doi:10.1007/s10555-012-9387-3 (2012).

83 Fan, T., Jiang, S., Chung, N., Alikhan, A., Ni, C., Lee, C. C. & Hornyak, T. J. EZH2-dependent suppression of a cellular senescence phenotype in melanoma cells by inhibition of p21/CDKN1A expression. *Molecular cancer research : MCR* **9**, 418-429, doi:10.1158/1541-7786.MCR-10-0511 (2011).

84 Rush, M., Appanah, R., Lee, S., Lam, L. L., Goyal, P. & Lorincz, M. C. Targeting of EZH2 to a defined genomic site is sufficient for recruitment of Dnmt3a but not de novo DNA methylation. *Epigenetics : official journal of the DNA Methylation Society* **4**, 404-414 (2009).

85 Esteller, M. CpG island methylation and histone modifications: biology and clinical significance. *Ernst Schering Research Foundation workshop*, 115-126 (2006).

86 Deaton, A. M. & Bird, A. CpG islands and the regulation of transcription. *Genes & development* **25**, 1010-1022, doi:10.1101/gad.2037511 (2011).

87 Fan, S., Zhang, M. Q. & Zhang, X. Histone methylation marks play important roles in predicting the methylation status of CpG islands.

Biochemical and biophysical research communications **374**, 559-564, doi:10.1016/j.bbrc.2008.07.077 (2008).

88 Caputo, V. S., Costa, J. R., Makarona, K., Georgiou, E., Layton, D. M., Roberts, I. & Karadimitris, A. Mechanism of Polycomb recruitment to CpG islands revealed by inherited disease-associated mutation. *Human molecular genetics* **22**, 3187-3194, doi:10.1093/hmg/ddt171 (2013).

89 Sahin, M., Sahin, E., Gumuslu, S., Erdogan, A. & Gultekin, M. DNA methylation or histone modification status in metastasis and angiogenesis-related genes: a new hypothesis on usage of DNMT inhibitors and S-adenosylmethionine for genome stability. *Cancer metastasis reviews* **29**, 655-676, doi:10.1007/s10555-010-9253-0 (2010).

90 Yu, M., Mazor, T., Huang, H., Huang, H. T., Kathrein, K. L., Woo, A. J., Chouinard, C. R., Labadorf, A., Akie, T. E., Moran, T. B., Xie, H., Zacharek, S., Taniuchi, I., Roeder, R. G., Kim, C. F., Zon, L. I., Fraenkel, E. & Cantor, A. B. Direct recruitment of polycomb repressive complex 1 to chromatin by core binding transcription factors. *Molecular cell* **45**, 330-343, doi:10.1016/j.molcel.2011.11.032 (2012).

91 Bracken, A. P., Pasini, D., Capra, M., Prosperini, E., Colli, E. & Helin, K. EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. *The EMBO journal* **22**, 5323-5335, doi:10.1093/emboj/cdg542 (2003).

92 Friedman, J. M., Jones, P. A. & Liang, G. The tumor suppressor microRNA-101 becomes an epigenetic player by targeting the polycomb group protein EZH2 in cancer. *Cell cycle* **8**, 2313-2314 (2009).

93 Varambally, S., Cao, Q., Mani, R. S., Shankar, S., Wang, X., Ateeq, B., Laxman, B., Cao, X., Jing, X., Ramnarayanan, K., Brenner, J. C., Yu, J., Kim, J. H., Han, B., Tan, P., Kumar-Sinha, C., Lonigro, R. J., Palanisamy, N., Maher, C. A. & Chinnaiyan, A. M. Genomic loss of microRNA-101 leads to overexpression of histone methyltransferase EZH2 in cancer. *Science* **322**, 1695-1699, doi:10.1126/science.1165395 (2008).

94 Lobo, N. A., Shimono, Y., Qian, D. & Clarke, M. F. The biology of cancer stem cells. *Annual review of cell and developmental biology* **23**, 675-699, doi:10.1146/annurev.cellbio.22.010305.104154 (2007).

95 Federici, G., Espina, V., Liotta, L. & Edmiston, K. H. Breast cancer stem cells: a new target for therapy. *Oncology* **25**, 25-28, 30 (2011).

96 Tieu, K. S., Tieu, R. S., Martinez-Agosto, J. A. & Sehl, M. E. Stem cell niche dynamics: from homeostasis to carcinogenesis. *Stem cells international* **2012**, 367567, doi:10.1155/2012/367567 (2012).

97 Singh, S. R. Stem cell niche in tissue homeostasis, aging and cancer. *Current medicinal chemistry* **19**, 5965-5974 (2012).

98 Hoggatt, J. & Scadden, D. T. The stem cell niche: tissue physiology at a single cell level. *The Journal of clinical investigation* **122**, 3029-3034, doi:10.1172/JCI60238 (2012).

99 Scaffidi, P. & Misteli, T. Cancer epigenetics: from disruption of differentiation programs to the emergence of cancer stem cells. *Cold Spring Harbor symposia on quantitative biology* **75**, 251-258, doi:10.1101/sqb.2010.75.007 (2010).

100 Baylin, S. B. in *StemBook* (2008).

101 Lotem, J. & Sachs, L. Epigenetics and the plasticity of differentiation in normal and cancer stem cells. *Oncogene* **25**, 7663-7672, doi:10.1038/sj.onc.1209816 (2006).

102 Simon, J. A. & Lange, C. A. Roles of the EZH2 histone methyltransferase in cancer epigenetics. *Mutation research* **647**, 21-29, doi:10.1016/j.mrfmmm.2008.07.010 (2008).

103 Kaneko, S., Li, G., Son, J., Xu, C. F., Margueron, R., Neubert, T. A. & Reinberg, D. Phosphorylation of the PRC2 component Ezh2 is cell cycle-regulated and up-regulates its binding to ncRNA. *Genes & development* **24**, 2615-2620, doi:10.1101/gad.1983810 (2010).

104 Zeng, X., Chen, S. & Huang, H. Phosphorylation of EZH2 by CDK1 and CDK2: a possible regulatory mechanism of transmission of the H3K27me3 epigenetic mark through cell divisions. *Cell cycle* **10**, 579-583 (2011).

105 Palacios, D., Mozzetta, C., Consalvi, S., Caretti, G., Saccone, V., Proserpio, V., Marquez, V. E., Valente, S., Mai, A., Forcales, S. V., Sartorelli, V. & Puri, P. L. TNF/p38 alpha/Polycomb Signaling to Pax7 Locus in Satellite Cells Links Inflammation to the Epigenetic Control of

Muscle Regeneration. *Cell stem cell* **7**, 455-469, doi:DOI 10.1016/j.stem.2010.08.013 (2010).

106 Wei, Y., Chen, Y. H., Li, L. Y., Lang, J., Yeh, S. P., Shi, B., Yang, C. C., Yang, J. Y., Lin, C. Y., Lai, C. C. & Hung, M. C. CDK1-dependent phosphorylation of EZH2 suppresses methylation of H3K27 and promotes osteogenic differentiation of human mesenchymal stem cells. *Nature cell biology* **13**, 87-94, doi:10.1038/ncb2139 (2011).

107 Cha, T. L., Zhou, B. P., Xia, W., Wu, Y., Yang, C. C., Chen, C. T., Ping, B., Otte, A. P. & Hung, M. C. Akt-mediated phosphorylation of EZH2 suppresses methylation of lysine 27 in histone H3. *Science* **310**, 306-310, doi:10.1126/science.1118947 (2005).

108 Malumbres, M. & Barbacid, M. Cell cycle, CDKs and cancer: a changing paradigm. *Nature reviews. Cancer* **9**, 153-166, doi:10.1038/nrc2602 (2009).

109 Oshima, J. & Campisi, J. Fundamentals of cell proliferation: control of the cell cycle. *Journal of dairy science* **74**, 2778-2787, doi:10.3168/jds.S0022-0302(91)78458-0 (1991).

110 Johansson, M. & Persson, J. L. Cancer therapy: targeting cell cycle regulators. *Anti-cancer agents in medicinal chemistry* **8**, 723-731 (2008).

111 Giordano, A., Bellacchio, E., Bagella, L. & Paggi, M. G. Interaction between the Cdk2/cyclin A complex and a small molecule derived from the pRb2/p130 spacer domain: a theoretical model. *Cell cycle* **6**, 2591-2593 (2007).

112 Miranda, T. B., Cortez, C. C., Yoo, C. B., Liang, G., Abe, M., Kelly, T. K., Marquez, V. E. & Jones, P. A. DZNep is a global histone methylation inhibitor that reactivates developmental genes not silenced by DNA methylation. *Molecular cancer therapeutics* **8**, 1579-1588, doi:10.1158/1535-7163.MCT-09-0013 (2009).

113 Kikuchi, J., Takashina, T., Kinoshita, I., Kikuchi, E., Shimizu, Y., Sakakibara-Konishi, J., Oizumi, S., Marquez, V. E., Nishimura, M. & Dosaka-Akita, H. Epigenetic therapy with 3-deazaneplanocin A, an inhibitor of the histone methyltransferase EZH2, inhibits growth of non-small cell lung cancer cells. *Lung cancer* **78**, 138-143, doi:10.1016/j.lungcan.2012.08.003 (2012).

114 Puppe, J., Drost, R., Liu, X., Joosse, S. A., Evers, B., Cornelissen-Steijger, P., Nederlof, P., Yu, Q., Jonkers, J., van Lohuizen, M. & Pietersen, A. M. BRCA1-deficient mammary tumor cells are dependent on EZH2 expression and sensitive to Polycomb Repressive Complex 2-inhibitor 3-deazaneplanocin A. *Breast cancer research : BCR* **11**, R63, doi:10.1186/bcr2354 (2009).

115 McCabe, M. T., Ott, H. M., Ganji, G., Korenchuk, S., Thompson, C., Van Aller, G. S., Liu, Y., Graves, A. P., Della Pietra, A., 3rd, Diaz, E., LaFrance, L. V., Mellinger, M., Duquenne, C., Tian, X., Kruger, R. G., McHugh, C. F., Brandt, M., Miller, W. H., Dhanak, D., Verma, S. K., Tummino, P. J. & Creasy, C. L. EZH2 inhibition as a therapeutic strategy

for lymphoma with EZH2-activating mutations. *Nature* **492**, 108-112, doi:10.1038/nature11606 (2012).

116 Flemming, A. Deal watch: Epizyme-Celgene deal signals interest in new class of epigenetic drugs. *Nature reviews. Drug discovery* **11**, 508, doi:10.1038/nrd3772 (2012).

117 McCarthy, A. Epizyme: plying the epigenome's enzymes. *Chemistry & biology* **18**, 403-404, doi:10.1016/j.chembiol.2011.04.005 (2011).

CHAPTER 7. VITA

Adam Michael LaBaff was born in Potsdam, New York on March 16, 1982. He is the older brother of Benjamin Wayne LaBaff and son of Wayne Michael LaBaff and Susan Elizabeth LaBaff. He entered Clarkson University and received a B.S. in Biomolecular Science in 2004. Adam returned to Clarkson University to study in the lab of Dr Edward Moczydlowski and Dr Jon-Paul Bingham in the field of neurobiology and solid-phase chemistry and obtained a M.S. degree in 2007 in chemistry and biomolecular science. In August of 2006 while finishing the writing of his M.S. thesis he enrolled in The University of Texas, Health Science Center at Houston, Graduate School of Biomedical Science. One year later he enrolled in The Cancer Biology Program and began to conduct his PhD. Research under the guidance of Professor Mien-Chie Hung in the Department of Molecular and Cellular Oncology at The University of Texas, M.D. Anderson Cancer Center. Adam was awarded the degree of Doctor of Philosophy in May 2014.